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BIOMARKERS OF EXPOSURE TO TOXIC SUBSTANCES
Volume VII:
Identification of Potential Serum Protein Biomarkers Indicative of Low Level Kidney Degradation in Response to Toxin Exposures

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14. ABSTRACT
Potential serum biomarkers to subclinical nephrotoxin exposures were evaluated based on differential protein expression between control and dosed samples in rat serum. Proteins of interest demonstrated up-regulation at a minimum 1.5 fold increase in protein concentration control versus dosed sample. In order to identify common biomarkers of kidney decrement, three nephrotoxicants were chosen: D-serine, Puromycin, an antibiotic that degrades and Bromoethyl Amine (BEA). Rats were dosed with individual nephrotoxins at levels previously determined, via hisopathology, to initiate low level renal damage. Blood (serum) was collected at pre-dose, 24 hours post dose, and terminal sacrifice for puromycin and BEA, and at 12 and 24 hours post dose from D-serine. Method development of the immunodepletion step demonstrated that the Proteomet Lab IgY-R7 column worked substantially better than the Agilent MARS (HPLC and Spin) columns in a rat sera matrix. The collected serum samples were immunodepleted using the IgY-R7 column, separated using 2D Difference in Gel Electrophoresis (DIGE), and screened for potential up-regulated biomarkers based on differences in fluorescent intensity via computer software analyses. Serum peptides were identified by MALDI-TOF/TOF mass spectroscopy and MASCOT database alignments. This study identified 32 serum proteins that were up-regulated in response to D-serine exposures.

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PREFACE

This research was accomplished by personnel at the Applied Biotechnology Branch, Human Effectiveness Directorate of the 711th Human Performance Wing (711 HPW/RHPB) of the Air Force Research Laboratory, Wright-Patterson AFB, OH, under Dr. John J. Schlager, Branch Chief. This technical report was written under AFRL Work Unit 7184D405.

All studies involving animals were approved by the Wright-Patterson Institutional Animal Care and Use Committee, and were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the *Guide for the Care and Use of Laboratory Animals, National Research Council* (1996). Studies were conducted under approved Air Force Research Laboratory Institutional Animal Care and Use Committee Protocol F-WA-2003-0074-A.

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SUMMARY

Potential serum biomarkers to subclinical nephrotoxin exposures were evaluated based on differential protein expression between control and dosed samples in rat serum. Proteins of interest demonstrated up-regulation at a minimum 1.5 fold increase in protein concentration control versus dosed sample. In order to identify common biomarkers of kidney decrement, three nephrotoxicants were chosen: 1) D-serine, which causes necrosis of the proximal straight tubules, 2) Puromycin, an antibiotic that degrades the Glomerular Basement Membrane (GBM), and 3) Bromoethyl Amine (BEA), which also affects the proximal tubules. Rats were dosed with the individual nephrotoxins at levels previously demonstrated via hisopathology to initiate low level renal damage. Blood (serum) was collected at pre-dose, 24 hours post dose, and terminal sacrifice for puromycin and BEA, and at 12 and 24 hours post dose from D-serine. Method development and optimization of the immunodepletion step demonstrated that the Proteomet Lab IgY-R7 column worked substantially better than the Agilent MARS (HPLC and Spin) columns in a rat sera matrix. The collected serum samples were immunodepleted using the IgY-R7 column, separated using 2D Difference in Gel Electrophoresis (DIGE), and screened for potential up-regulated biomarkers based on differences in fluorescent intensity via computer software analyses. Finally, up-regulated protein ‗spots' of interest were isolated, trypsin-digested, and the resultant peptides identified by MALDI-TOF/TOF mass spectroscopy and MASCOT database alignments. This study identified 32 proteins that were up-regulated in response to D-serine exposures, with a subset of two potential serum biomarkers indicative of low-level kidney injury due to nephrotoxin exposures. Due to sample pooling requirements attributable to sample quantity after serum depletion, no dose or time dependent toxicity response could be determined utilizing the 2D DIGE/MALDI-TOF/TOF protocol.

1. INTRODUCTION

1.1. Background

A major challenge for health care professionals is the detection and accurate diagnosis of early onset renal degradation. Traditionally, clinicians have determined kidney health by measuring blood urea nitrogen (BUN) in serum and creatinine levels in urine (Duarte and Preuss, 1993). Unfortunately, these biological markers may not be evident until kidney functions have decreased by 30% - 50%, as measured by histopathology functional tests, nor do these biomarkers pinpoint specific regions of damage (Thukral et al., 2005). At such late stages of kidney degradation, full recovery by a patient is rare and the outcome is often a lifetime of mitigating symptoms by dialysis treatments or, if lucky, a kidney transplant.

In general, a patient has a much better chance of full recovery if an accurate diagnosis is made early during onset of the damage. This is especially true when combating degenerative conditions like kidney failure. Standard methods of diagnosing a disease include visual diagnosis (cut or laceration), direct diagnosis (biopsy), and indirect diagnosis (biological markers, i.e. measurement of creatinine levels) (Veenstra et al., 2005). However, when diagnosing kidney diseases these tools are not effective simply because they are not available, too traumatic, or the marker response is too slow after kidney damage. Decreased renal function has no visible signs or pain and can only be diagnosed through direct and active medical diagnoses. While direct analysis of kidney tissue by biopsy can help diagnose specific ailments and direct therapeutic paths, clinical biopsies are not routine since they are invasive and can suffer from sampling errors (Sharma et al., 2005). Therefore indirect analysis has been the best tool for routinely testing the growing number of patients who suffer from deteriorating kidney function such as chronic kidney disease (CKD).

Chronic kidney disease demonstrates the need for improved renal diagnostic methods as it affects approximately 19 million people in America alone, yet often goes undiagnosed until it becomes too late for a patient to fully recover (Coresh, 2005). CKD

is a general decrease in renal function occurring over months or years and has no specific treatment. Often the clinical signs of the disease only manifest as a resulting complication like pericarditis or anemia before diagnostic measurements are taken. The current diagnostic methods look for proteinuria or elevated serum creatinine levels (Snyder and Pendergraph, 2005). Unfortunately both indicators are well documented as being too generic (Tomlanovich et al., 1986; Baboolal, 2002; Star, 1998) and can neither diagnose the type of renal injury nor be consistently reliable. For instance, serum creatinine is heavily influenced by many factors, such as muscle mass and tubular secretion, which means serum samples must be taken frequently over long periods of time to normalize results (Tomlanovich et al., 1986). New techniques utilizing proteomic research for diagnoses use biological fluid (biofluid) samples which can be analyzed for qualitative or quantitative changes in proteins that may indicate physiological injury.

The use of proteomics in biomarker discovery is well documented and consists of a wide array of dynamic techniques with updated and improved methodologies published weekly. A good example of these techniques successfully identifying useful protein biomarkers in a relatively short time to a new threat was published by Chen et al. (2004) and their examination of the Severe acute respiratory syndrome (SARS) virus. Chen et al. (2004) compared 22 different plasma samples from four SARS patients with plasma samples from healthy controls. Subsequent 2D PAGE analysis found seven distinct proteins in SARS patients that were absent in healthy controls as well as eight spots up-regulated in all 22 samples. One of the up-regulated spots, peroxiredoxin II, was found to be in all 22 samples and later validated in ~36% of SARS patients. This detection rate is higher than HIV testing and may be used as a future serum biomarker for SARS. Thanks to this research, the quest to identify a validated protein biomarker for SARS has moved on to the next stage of development. The work by Chen et al. (2004) demonstrated that biomarker research techniques can be used successfully to rapidly identify biomarkers for disease diagnosis even if the disease is new and not fully understood. The biomarker techniques used by Chen et al. are part of biomarker research in general which can be divided into two main themes, biomarker discovery or diagnostic development (Veenstra et al., 2005).

Biomarker discovery uses mass spectroscopy techniques to identify proteins and characterize peptide sequences. The aim in this approach is the identification of a change in relative abundance of peptides from a unique protein present in samples obtained from disease cases compared with matched controls (Veenstra et al., 2005). Diagnostic development is a comparative approach to identifying a disease state by looking at overall protein peak patterns on a mass spectrometer. This approach uses a large sample size from hundreds of patients to find statistical differences between protein peaks and then uses data mining with bioinformatic algorithms to find diagnostic protein peaks. However, the exact relation between the diagnostic proteins and the disease condition are unknown, since specific proteins are not identified (Veenstra et al., 2005). The proposed research will focus on biomarker discovery and the identification of specific proteins which may be used to track preclinical levels of kidney damage. In support of this mass spectroscopy based approach, this study will use 2D Difference in Gel Electrophoresis (DIGE) coupled with Matrix Assisted Laser Desorption Ion-Time of Flight/Time of Flight (MALDI-TOF/TOF) analysis of rat serum samples to specifically identify proteins as biomarkers of preclinical levels of kidney injury.

As of late, published proteomic results have focused on analyzing specific organ tissue with little published data using biological fluids like blood or urine. The search for protein biomarkers in blood implies a distinct interest for clinical applications since organ functions could be tested using blood samples, which are less invasive than tissue biopsies (Bandara et al., 2002). Published results show that protein expression can be characterized from biofluid samples such as serum, plasma, urine, and cerebrospinal fluid, as effectively as from diseased tissue samples (Pang et al., 2002; Perroud, et al. 2006). The abundance of proteins and relative ease of biofluid sample collection mean that self monitoring of health could become routine and diseases could be diagnosed before physical symptoms manifest. Although published results from biofluid analysis lags behind tissue analysis, proteomic techniques used in analyzing biofluids have made rapid advancements and clinical uses may be in the very near future (Veenstra et al., 2005).

Of the four main biofluids that are easily obtainable to researchers and clinicians alike, urine and serum samples are currently thought to be the best samples for examining kidney injury. While urine is a superior sample due to ease of collection, ample sample volume, and cost of analysis, this study will focus on analyzing serum. According to Sharma, proteomic studies using urine presents many challenges such as protein degradation during storage as well as fluctuations in protein levels depending on timing of collection, diet of subjects, and gender (Sharma et al., 2005). Additionally, urine samples face many of the same problems as plasma samples, namely abundant proteins such as IgG and albumin. In addition, Sharma also found that IgG leads to streaking of the 2D gels and that degraded albumin products may account for a large number of differentially regulated spots (Sharma et al., 2005).

The use of serum samples during this research project posed several challenges, primarily due to lack of published research on serum proteomic techniques. Veenstra found that 22 proteins, usually albumin, transferrins, immunoglobulins, and complement factors, make up 99% of all proteins in plasma and serum (Veenstra et al., 2005). The low abundant proteins are circulatory proteins from live, apoptotic, and necrotic cells, and the detection of these very low level proteins require sensitive quantitation to detect these protein differences in such a small set of diseased cells or in biofluids. Discovering the proteins derived from damaged cells specific to a disease becomes much more difficult as a disease progresses and affects nearby organs or cell layers. One such study by Pieper identified ~20,000 spots by image processing (Pieper, Gatlin, et al., 2003). Of these ~20,000 spots, about 3,700 unique spots were found once redundant images were eliminated. From the 3,700 spots, 1,800 were identified which yielded 350 unique proteins (Pieper, Su, et al., 2003). Of the 350 unique proteins Pieper et al. found, almost 39% have been previously characterized as circulatory proteins. Another 9% of the identified proteins were characterized as proteins which are secreted into biofluids other than blood. Very low abundant proteins such as IL-6, metallothionein II, cathepsins, and peptide hormones were also identified. These low abundant proteins are known to be in serum at concentrations less than 10 ng/ml (Pieper, Gatlin, et al. 2003). Therefore it is essential that the high abundant proteins be removed from the sample prior to any sample

analysis. To overcome the signal dilution by the abundant proteins, the use of an immunodepletion step prior to 2D gel separation technique has been developed to allow the quantitative measurement of proteins between samples while leaving a visual record of the many isoforms a protein can assume (Veenstra et al., 2005).

Pieper also performed a similar study using in a urine matrix which, while yielding good results, were not as complete the prior serum study. In this part of the study, the urinary proteome was characterized using a similar strategy of protein separation by 2D-PAGE followed by MS identification (Pieper, 2004). The samples went through similar preparation steps including an immunodepletion step (Pieper 2004, Pieper, 2008). The group found 1,400 distinct spots on gels and 420 proteins were identified by either MALDI-TOF peptide mass fingerprinting or LC-ESI MS/MS. Results identified 150 unique proteins, with ~50 known classical plasma proteins shown also present in urine (Veenstra et al., 2005). These results show that analyzing the serum produces more comprehensive results and can express more low abundant proteins since most of them will be transported by albumin and other transport proteins.

A study by Bandara et al. (2003) proved that kidney injury biomarkers can be identified using 2D gel separation followed by MS characterization. This study examined the response to d-serine as well as two other well characterized kidney toxins, cisplatin and 4-aminopyridine (4-AP). The d-serine results are of particular interest since it is one of the nephrotoxins studied in this project. Bandara et al. (2003) found 11 main proteins exhibiting changes in expression levels as a result of d-serine induced nephrotoxicity. Several of the 11 proteins were found to overlap with results from the cisplatin and 4-AP samples, like the cellular enzyme fumarylacetoacetate hydrolase (FAH) found in d-serine and 4-AP. Of the proteins expressed in all samples from all toxicants tested, three very significant proteins were identified as potential biomarkers for clinical diagnosis. The most promising of the three was a rat-specific protein known as T-kininogen. The most promising of the three proteins was a rat-specific protein known as T-kininogen. This protein was found to exhibit distinct time and dose dependencies and showed different concentration levels compared to control samples and samples from

rats dosed with L-serine. The time and dose dependencies are exemplified by measured increases in protein concentrations and by peak concentration level timing. Seven isoforms of T-kininogen were found, six in the d-serine samples and one from the 4-AP and cisplatin samples. The isoform concentrations were found to increase directly with the extent of the observed cellular damage, thus demonstrating the dose-response relationship of d-serine toxicity. The peak concentration of T-kininogen occurred at the 24 hour period which correlated with the damage exhibited by the proximal tubule tissue. Peak kidney damage, as indicated by BUN and plasma creatinine levels, was also observed at 24 hours and subsequently improved as the kidneys returned to normal function over a three-week period post exposure.

There are three classes of kininogens: T-, H- and L-kininogen. Both H- and L-kininogen are expressed in both rodents and humans, whereas T-kininogen is only expressed in rats (Greenbaum et al., 1992; Takano et al., 1997). The liver synthesizes all three kininogen isoforms but studies have also found the kininogen species to be secreted into blood. Kininogens play an important part in the release of bradykinin, a well-documented peptide hormone, by acting as a substrate for the kallikrein serine proteases. Kallikrein serine proteases cleave proteins to release bradykinin (Bandara, Kelly, et al. 2003; Bandara and Kennedy 2002).

Also of interest from Bandara et al. was the identification of retinol binding protein (RBP) in the d-serine study as RBP has previously been linked to renal toxicity (Bandara, Kelly, et al. 2003). The regulation of RBP was treatment-specific – the changes were not observed in control animals or the L-serine-treated group. Although RBP is required for the transport of vitamin A in blood, it is also reabsorbed and degraded in the renal proximal tubules (Goodman, 1984). Several reports have focused on the elevated presence of RBP in the urine of patients or animals with renal damage (Brouwer et al., 1988; Brouwer, et al. 1989; Jung et al., 1993). The identification of RBP is important as it validated the capacity of 2D DIGE proteomics to identify new biomarkers of toxicity by its detection of known kidney injury marker proteins. Given the extensive work done by Bandara, it was expected that T-kininogen and RBP would be identified as biomarkers in

this study as well - the primary difference between the published Bandara research and this study is in the use of the more sensitive of 2D DIGE technology instead of the standard 2D gels.

1.2. Kidney Anatomy and Physiology

The kidney is a robust organ designed to filter waste products from the bloodstream and excrete them from the body (Figure 1).

Since nearly 25% of the cardiac output goes directly to the kidney, it becomes extremely susceptible to toxic injury from xenobiotics and drugs. Xenobiotics and drugs are metabolized and concentrated in the kidney from the blood stream, placing potentially harmful substances into one localized area and thereby increasing potential tissue damage within the parenchyma (Toback, 1992; Bennett, 1997).

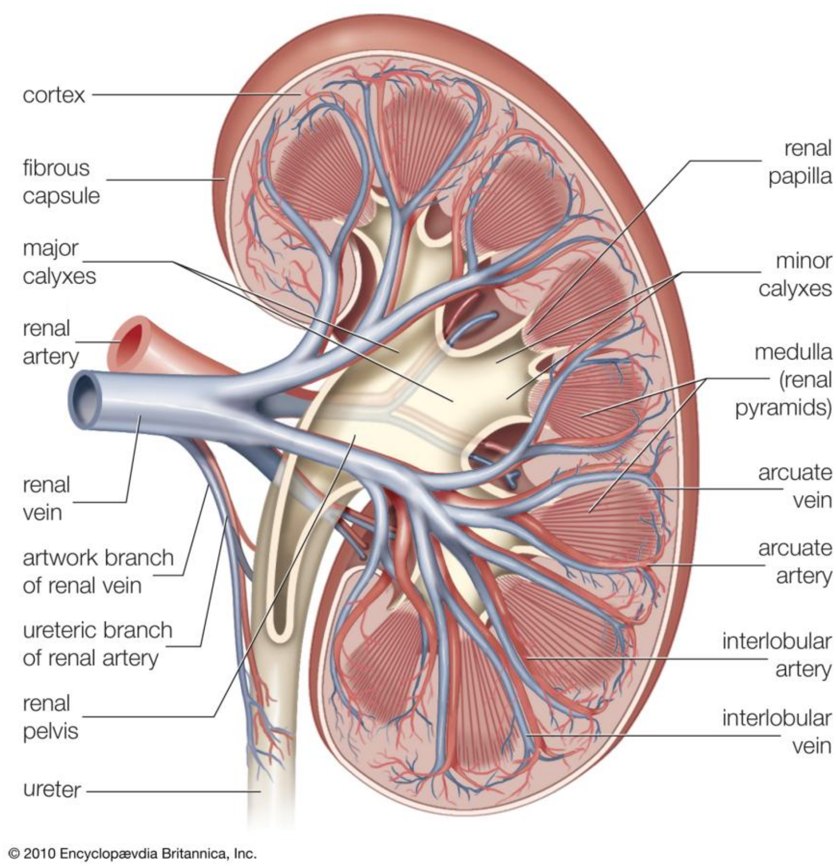


Figure 1: Cross section of the Kidney (*taken from Encyclopaedia Britannica, Inc.).*

In the circulatory system about 25% of blood volume flows directly from the heart to the kidney where it is dispersed and filtered by tissue called nephrons. Collectively these nephrons make up the parenchyma (the functional bulk of the organ). Within each nephron there exists a complex system of structures designed to filter the blood, reabsorb necessary compounds, and excrete wastes as urine.

The first structure to filter blood is the Renal Corpuscle which consists of the Glomerulus and Bowman's capsule. The glomerulus is a capillary tuft which receives blood from the afferent arteriole and sends filtered blood to the renal vein for further processing. Bowman's capsule (glomerulus capsule) surrounds the glomerulus, is made of epithelial cells, and works with the glomerulus to act as the first filter of the nephron.

The renal tubule is made up of the proximal tubule, the loop of Henle, and the distal convoluted tubule (Figure 2). The proximal tubule, especially along the straight, descending portion, is the area of primary interest when preventing or treating kidney failure. The proximal straight tubules are responsible for regulating re-absorption of fluids like salt and water, as well as organic solutes like glucose and amino acids, into the peritubular capillaries. Because of the steady filtering and waste concentration, these cells are constantly exposed to toxic xenobiotics or metabolites at concentrations much higher than other cells are typically exposed. The constant filtering and re-absorption means that in the kidney, the renal tubular epithelial cells are the most susceptible to toxic injury. The health and status of the glomerulus and renal proximal tubules are the major concerns when diagnosing kidney health, therefore a range of nephrotoxins in this study were selected based on their differential kidney segment effects.

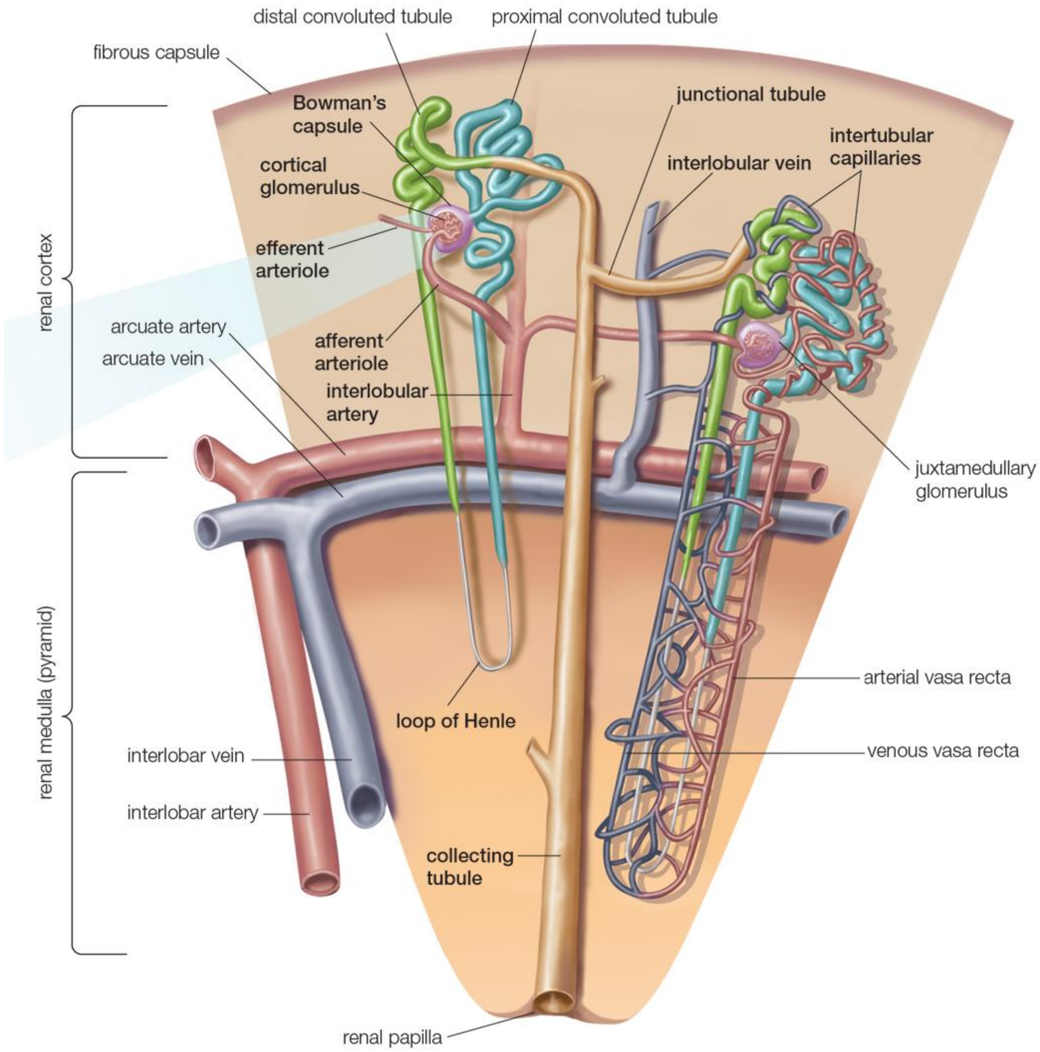


Figure 2: Image of filtration tubules in rat kidney *(taken from Encyclopædia Britannica, Inc.)*

1.3. Nephrotoxin Background

1.3.1 D-Serine

D-Serine is a well characterized nephrotoxin that has has been shown to cause necrosis of the proximal straight tubules in the rat kidney (Figure 3). The precise mechanism of toxicity remains unclear, although d-serine is known to concentrate in the damaged areas of the nephron (Imai et al., 1998). The d-serine is re-absorbed into the proximal tubules where it enters cells in the pars recta and becomes processed by d-

amino oxidase (Silbernagl et al., 1999). The reaction with the d-amino oxidase enzyme produces toxic oxidative metabolites leading to necrosis of the epithelium cells lining the proximal tubules. The damage to the tubules results in glucosuria, diuresis, aminoaciduria, and proteinuria (Ganote et al., 1974). A histopathological study by Ganote et al. (1974) examined sections of rat kidney by light and electron microscopy and found that proteinuria and glucosuria are caused by the diffusion of protein and glucose from interstitial fluid to tubular fluid across the necrotic tubular epithelium. Ganote et al. (1974) observed the stages of necrosis and recovery over a period of 6 days, and noted that the cells showed shrinkage during initial stages, followed by either immediate lysis of the nuclear contents or swelling and loss of apical cytoplasm. Without the tubular epithelium the proteins and glucose concentrated in the interstitium, freely crossing from the interstitial to the luminal fluids through the denuded glomerular basement membrane.

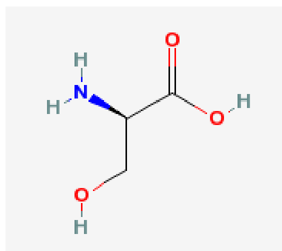


Figure 3: Stick model of d-serine

1.3.2 Puromycin

Puromycin aminonucleoside (PAN) is an antibiotic that is often used to study glomerular injury (Figure 4). PAN causes the glomerular epithelial cells (GEC) to flatten which leads to a loss of filtration function as well as loss of epithelial cells along the glomerular basement membrane (GBM) ultimately resulting in proteinuria (Fishman and Kamoysky, 1985).

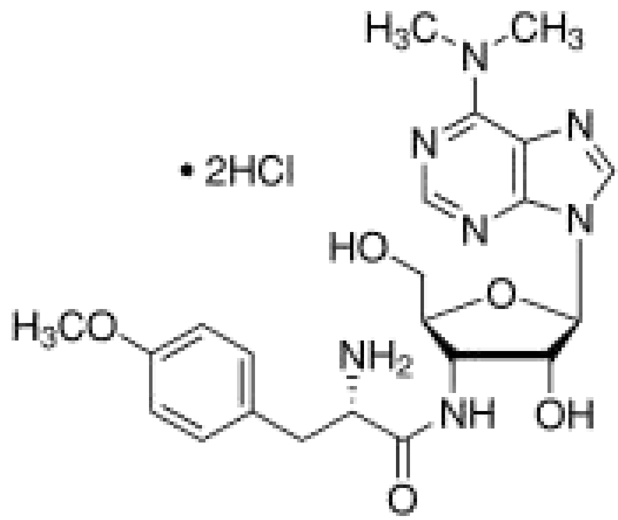


Figure 4: Model of Puromycin

The normal glomerular capillary wall keeps blood cells and most proteins in the blood but in patients with proteinuria, cellular degeneration allow proteins to leak across the glomerular wall into the urine. The barriers in the capillary wall that keep protein out of the urine are the endothelial cells lining the capillary lumen, the basement membrane under the endothelial cells and the cells on the outside surface of the capillary (epithelial cells). The epithelial cells (podocytes) normally have little ―feet" (pods) that sit on the basement membrane, connected by a thin membrane. The glomerular epithelial cells (podocytes) appear to be the most important barrier that prevents protein from leaking into the urine (Farquhar, 1975).

A study by Ryan and Karnovsky (1975) found that focal detachment of the GEC from the GBM occurred at the same time that massive proteinuria was measured. Additionally, it was observed that areas stripped of GEC corresponded to an increase of ferritin leakage, a well-known proteinuria marker, across the GBM. This leakage suggests that GEC detachment is a contributing factor to proteinuria in the PAN-induced nephrosis model.

In the study by Sanwal et al. (2001), plated cells sustained in media were dosed with 5, 10, 20, 50, 100, 200, and 500 ug/ml of puromycin. Morphologic analysis using

fluorescence staining showed that PAN enhanced GEC apoptosis in a time- and dose-dependent manner at 24 and 48 h and only induced necrosis of GEC at high concentrations. Signs of necrosis were initiated at 100 ug/ml, but PAN induced the greatest amount of damage at 24 h with doses of 200 ug/ml and 500 ug/ml. Since PAN induced necrosis at higher concentrations and apoptosis at lower concentrations in a concentration-dependent manner, the data suggests that the severity of cellular injury determines whether cells die by necrosis or apoptosis (Sanwal et al., 2001).

The mechanism by which PAN initiated apoptosis and necrosis of GECs is unclear, but it is known that hydrolysis of puromycin creates PAN. PAN, which is structurally similar to adenosine, inhibits *de novo* protein synthesis and can potentially prevent synthesis of nucleic acids. *In vitro* studies demonstrate that PAN causes premature chain termination by acting as an analog of the 3'-terminal end of aminoacyl-tRNA (de la Luna and Ortin, 1992) and can attenuate *de novo* RNA synthesis and DNA synthesis in a variety of cells (Sanwal et al., 2001). Additionally, free radicals have been implicated in causing kidney injury after exposure to PAN. Diamond et al. found that the xanthine oxidase pathway creates reactive oxygen species and may ultimately be the mechanism of nephrosis (Diamond et al., 1986).

1.3.3 Bromoethylamine (BEA)

Alkylhalide 2-bromoethylamine hydrobromide (BEA) produces renal injury in rats and mimics analgesic-related renal injury in humans (Figure 5) (Hedlund et al., 1991). 2-Bromoethylamine hydrobromide (BEA) is a low molecular weight halogenated chemical which causes reproducible lesions in the renal papilla of experimental animals (Murray et al., 1971). Histologically, BEA produces necrosis of the thin ascending limb of the loop of Henle and collecting duct within 6 hrs of dosing followed by complete papillary necrosis in 7 to 14 days (Murray et al., 1971). Functionally, BEA causes impaired urinary concentrating ability as well as a loss of sodium, chloride, and under certain circumstances potassium (Arruda et al, 1979; Sabatini et al., 1981). There is also an apparent loss in the proportion of filtering juxtamedullary nephrons (Sabatini et al., 1981). Although the molecular mechanism of BEA-induced papillary necrosis is

unknown, maintenance of the medullary concentration gradient is requisite to the development of papillary necrosis (Sabatini et al. 1983). It is apparent that the physiological function of the renal medulla to sustain an osmotic gradient makes this portion of the nephron uniquely susceptible to damage to agents such as BEA.

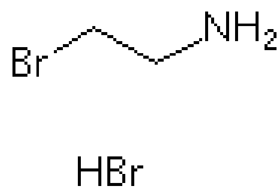


Figure 5: Model of Bromoethyl amine

1.4. Theory and Uses of Utilized Techniques and Instrumentation

1.4.1 Two-Dimensional Difference Gel Electrophoresis (2D DIGE)

In the search for novel biomarkers, accurate and precise isolation of proteins are crucial to identifying small, low abundant proteins that may be up- or down-regulated as a direct result of tissue degradation. One of the best techniques to visualize general protein expression levels is two-dimensional (2D) gel electrophoresis. A key concern regarding the use of 2D electrophoresis is the gel to gel reproducibility of the protein separations. In general, polyacrylamide gel separations are subject to internal errors from non-homogenous polymerization, temperature differentials, and fluctuations in electrical power (Speicher, 2004). These variables create slight differences between gels which make it difficult to match protein spots consistently across multiple gels. Inconsistent protein patterning requires powerful analytical software to examine thousands of protein spots to accurately identify identical and overlapping proteins across gels to measure the changing concentration. Thus, a high level of skill is needed to pour gels, run samples, and analyze spots to increase the statistical significance of the data (Speicher, 2004).

2D Difference Gel Electrophoresis represents an improvement over typical 2D gels because two samples and an internal standard can be all tagged with fluorescent dyes

and run in the same gel at the same time. In a DIGE gel, a control sample and a disease sample are labeled with two different fluorescent tags called Cy Dyes. By running both samples in the same gel, a more accurate comparison of protein size (based on location in the gel) and relative quantities (based on fluorescent intensity) can be made without gel to gel variability. Additionally, DIGE gels allow more accurate comparisons between different gels since an internal standard of all pooled samples is labeled with a third dye and added to every gel.

The first improvement lies in direct comparison between samples. The dyes allow two different sample groups to be labeled, mixed together, and separated on the same gel so they are exposed to identical electrophoretic conditions and gel inconsistencies. Any proteins present in both samples will migrate to the same areas of the gel and the different fluorescence spectra will indicate any definitive overlap. This accuracy reduces any technical variation, which may be as high as 20–30% (Molloy et al., 2003). The second improvement is the ability to more accurately compare samples between gels despite the variation that inherently exists in each individual gel. This feature is possible because of the internal pooled standard labeled with the third dye which is added to every gel. The pooled standard is crucial because it is made of equal parts from every sample thus contains every protein. By applying this standard to every gel, it provides a known landmark in each gel that is used to align multiple gels. This known correction factor gives a greater degree of confidence that protein expression differences and location are real, and not solely explained by chance or sample artifact (Hoorn et al., 2006).

The dyes used in this method are N-hydroxyl succinimidyl ester derivatives of Cy2, Cy3, and Cy5 and each dye has its own excitation and emission spectra. The dyes form an amide by reacting with the ε-amine group on lysine residues by nucleophilic substitution (Speicher, 2004). Since the dyes react with a polar amino acid each has a positive charge to replace the charge lost from the lysine and thus the original isoelectric point of each protein is retained (Speicher, 2004). The sensitivity of this technique depends on the technique used to label the sample with the fluorescent CyDyes. The two

labeling methods are called ―minimal” and ―saturation”. Minimal labeling uses the CyDyes and provides a detection limit between 150 and 500 pg (Hoorn et al., 2006). Saturation labeling uses thiol-reactive maleimide groups to react with cysteine residues and has been shown to have a lower limit of detection than minimal labeling (Shaw et al., 2003). While the saturation method is more sensitive, it is more technically challenging and is normally used only when samples are not limited (Lilley and Friedman, 2004).

The end result of the 2D DIGE technique allows direct comparison of two samples in one gel or multiple samples from multiple gels with minimal requirements for digital or statistical corrections for gel variations and warping. This technology was recently validated by Tonge et al. (2001) with proteomic studies on mouse liver, Zhou et al. (2002) with the identification of esophageal scans of cell cancer-specific protein markers, and Gharbi et al. (2002) analyzing the effects of the hepatotoxin, N-acetyl-p-aminophenol, on mouse liver protein expression.

1.4.2 DIGE Experimental Limitations

While 2D DIGE is an excellent tool for proteomic studies, it is not without its limitations. The first significant obstacle is developing the optimal conditions for protein separation. Tissue and biofluids are incredibly complex matrices that contain numerous interfering substances like proteases, salts, and nucleic acids which must be removed or inactivated (Hoorn et al., 2006). Removing these substances requires careful methods like desalting techniques or DNA/RNA nuclease treatments (Hoorn et al., 2006). Additionally, studies that search for small or low abundant proteins in blood must include an immunodepletion step before running the gel to remove large, over abundant carrier proteins like albumin, transferrins, and IgG. These proteins mask smaller proteins and the resultant huge smears in the gel make analysis impossible. Therefore, the immunodepletion step is especially crucial since serum samples contain a large number of proteins that range in concentrations by more than 10 orders of magnitude (Corzett et al., 2006). Though conditions for protein separation can be optimized, the DIGE technique still has physical limitations using current protocols.

According to Hoorn et al. (2006), the two major limitations to DIGE-based proteomics are the identification of low-abundant proteins and hydrophobic proteins. Issues with low abundant protein identification are the capabilities of visually isolating and identifying small spots, preparing them using many steps, and having enough stable protein to run through a mass spectrometer. Hoorn et al. (2006) have found that even optimized systems often do not exceed mass spectrometry identification rates of 50%. The difficulty with hydrophobic proteins, such as integral membrane proteins, are its solubility characteristics. Strong detergents like sodium dodecyl sulfate (SDS) are required to suspend integral membrane proteins in solution but such chemicals interfere with isoelectric focusing (Hoorn, et al. 2008; Hoorn, et al. 2006; Hoorn et al. 2005). The use of chaotropic agents like thiourea with urea or zwitterionic detergents like 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) help with solubilization without IEF interference (Hoorn, Hoffert et al. 2006). Another solution is the separation of proteins based on molecular weight using two runs. The first run uses a cationic detergent benzyldimethyl- n –hexadecylamonnium chloride while the second run uses SDS. Macfarlane et al. (1989) showed that proteins migrate differently using these two detergents which helps to maintain high resolution while keeping hydrophobic proteins solubilized.

Hoorn et al. created an excellent summary of the strengths and limitations of the DIGE technique as follows:

Strengths of DIGE-based proteomics

- Currently the only routine platform for quantitative proteomics
- Differential expression pattern with statistical output
- Low experimental variation due to mixing of experimental and control sample
- and inclusion of an internal standard
- Visualization of protein isoforms including splice variants and post-translational
- modifications

- Information on molecular weight and iso-electric point

Limitations of DIGE-based proteomics

- Less efficient for hydrophobic proteins (e.g., integral membrane proteins)
- Low identification rate of low abundance proteins
- Potential false positives using traditional statistics
- Difficult quantification and identification of proteins with overlapping spots

Possible solutions to overcome limitations

- Use of other detergents during iso-electric focusing
- Modified 2-DE (using 16-BAC and SDS)
- Modified statistical procedures
- Combination of targeted proteomics and DIGE-based proteomics
- Combination of DIGE-based proteomics with bioinformatics pathways analysis

1.4.3 Mass Spectrometry

At a basic level, mass spectrometry can be described as the study of gas-phase ions. The careful manipulation and measurements of the gas-phase ions generate practical data which can be used to characterize molecular structures (Kinter and Sherman, 2000). In a mass spectrometer, a sample is energized by an ion source, separated by its mass to charge (m/z) ratio in a mass analyzer, picked up by a detector and presented visually as a mass spectrum (Skoog et al., 1998). There are several types of ion sources and mass analyzers available, each with specific strengths and weaknesses which can be combined depending on the characteristics of the study sample. For biological molecules in general, publications indicate that a Matrix Assisted Laser Desorption / Ionization (MALDI) ion source coupled with a Time-Of-Flight (TOF) mass analyzer produces highly sensitive, accurate, and precise mass spectra. The spectra can be further analyzed using Tandem Mass Spectrometry to obtain protein and peptide sequences.

1.4.4 Matrix Assisted Laser Desorption/Ionization (MALDI)

Matrix-Assisted Laser Desorption/Ionization (MALDI) was introduced in 1988 by Karas and Hillenkamp as another technique for separation of high molecular weight biomolecules such as proteins and peptides (Kinter and Sherman, 2000). The MALDI ion source works by dissolving peptides in a solution of a UV-absorbing compound, called the ―matrix”, and placing the sample on a plate. As the solvent dries, the matrix compound crystallizes and peptide molecules are incorporated into matrix crystals. A UV laser light then vaporizes small amounts of the matrix /peptide ions, which are then carried into the gas phase. Ionization occurs by protonation in the acidic environments produced by the acidity of most matrix compounds and by the addition of dilute acid to the samples. Because the laser desorption generates ions in discreet, short packets, MALDI is ordinarily combined with time-of-flight (TOF) mass analysis. By combining MALDI with TOF analysis, two main benefits are achieved: very high sensitivities due to efficient protonation and high tolerance levels for contaminants. This high tolerance means that once a protein is digested, a MALDI spectrum can be obtained without further need for separation techniques like HPLC. By allowing for direct analysis of enzymatic digest analysis, MALDI is one of the fastest, most efficient means of ionizing and analyzing large biological molecules. (Kinter and Sherman, 2000).

1.4.5 Mass Analyzers

After a sample is ionized, the next step in mass spectrometry is the measurement of the mass to charge ratio (m/z) via mass analyzer. The sample ions are split into two general categories: molecular ions, which contain the entire analyte molecule, and fragment ions, which contain only a portion of the structure (Kinter and Sherman, 2000). The molecular ion gives the molecular weight of the analyte while the analyte fragment ions give structural information. There are a wide variety of mass analyzers capable of making the necessary measurements such as quadrupole mass filter, ion trap, time-of-flight, magnetic sector, ion cyclotron resonance, among others (Kinter and Sherman, 2000). The nature of the mass analyzer determines several characteristics of the overall MS experiment and the two most important are m/z resolution (often called ―mass

resolution") and the m/z range of ions that can be measured (often called –mass range") (Kinter and Sherman, 2000).

The factors that determine mass accuracy include fundamental parameters such as the m/z resolution used when making the measurement, type of mass analyzer used, and how the data were recorded, as well as practical parameters such as the quality of the instrument calibration (Kinter and Sherman, 2000). Assuming proper calibration, quadrupole and ion trap mass analyzers operated at unit resolution will provide mass accuracies in the 100- to 200-ppm range (Kinter and Sherman, 2000). Time-of-flight instruments using delayed extraction and reflectron ion optics with high data-acquisition speeds to give an m/z resolution greater than 10,000 provide mass accuracies in the 5- to 20-ppm range (Kinter and Sherman, 2000).

1.4.6 TOF Mass Analyzers

The Time-Of-Flight mass analyzer is a very simple yet incredibly effective and robust system. A TOF analyzer first creates an electric field produced from a very high potential, typically +20 kV to +30 kV (Kinter and Sherman, 2000). Then, an analyte ion is accelerated through the field which imparts a fixed amount of kinetic energy. Once accelerated, the ion enters a field-free region where it travels at a velocity inversely proportional to its m/z (Kinter and Sherman, 2000). Due to the inverse proportionality, light ions with a low m/z move faster than heavy ions with a high m/z. Therefore, the time required for an ion to travel through the field-free region is measured and this measurement can be used to back calculate the velocity (Kinter and Sherman, 2000), which ultimately allows one to calculate the m/z, using the equation:

$$(V)z = \frac{1}{2} m v^2$$

Where (V) = kinetic energy in J, z = charge, m = mass in kg, and v = velocity in m/sec. The equation can be rearranged to give the velocity of the ion:

$$v = ((2Vz)/m)^{1/2}$$

The most important factors that determine resolution include details of the ionization process such as the time span of the ionization event and the energetics of the ions produced; the instrument dimensions, particularly the length of the flight tube; and the accelerating voltage used (Kinter and Sherman, 2000). While TOF mass analysis can produce accurate data due to the many advantages discussed previously, a single TOF analysis can only yield molecular weights since MALDI is a –soft” ionizing source, meaning analytes are ionized with little to no degradation in structure. In order to identify the proteins of interest, actual peptides were sequenced from the fragment data. To accomplish this, analyte ions were split and the m/z ratio measured using tandem mass spectrometry.

1.4.7 Tandem Mass spectrometry

In tandem mass spectrometry, an analyte ion is examined in two stages in one experiment. The first stage is to separate parent ions by m/z and the second stage is to isolate and fragment the analyte in order to determine the m/z of the daughter ions (Kinter and Sherman, 2000). Tandem mass spectrometers typically use –collisionally activated dissociation” (CAD) or –collisionally induced dissociation” (CID) which means that a mass-selected ion is hit with high energy gas molecules in a high pressure region. When the gas molecules collide with the ion, energy is transferred to the ion making it unstable and driving fragmentation reactions that occur prior to leaving the collision cell (Kinter and Sherman, 2000). After fragmenting, the daughter ions produced are m/z analyzed in the second stage of mass analysis based on TOF similar to the first stage of analysis (Kinter and Sherman, 2000).

1.4.8 Interpretation of product ion spectra

The product ion spectrum is interpreted and the peptide is sequenced by mathematically matching it to a finite, albeit large, set of possible amino acid sequences from protein sequence databases (Kinter and Sherman, 2000). By defining the set of possible amino acid sequences as the experimentally determined protein and gene sequences, the source protein sequence is also identified. Ideally, a protein is identified when a significant number of peptides can be matched to peptides derived from a particular database amino acid sequence (Kinter and Sherman, 2000). While database

mining can provide accurate peptide and protein identifications at incredible rates, the method does have drawbacks. Namely, mathematically matching spectra becomes statistical probability rather than a positive identification from direct methodical sequencing. The problem with statistical matching comes from the lack of absolute rules for judging significance of those scores beyond an intra-set ranking of possible matches between assigned scores and database information (Kinter and Sherman, 2000). It is also particularly difficult to judge cut-off values for the incorrect matches, typical minimal cut-off scores for matches are ion scores of 100 and peptide ion scores of 50. Additionally, until every protein possible is known, computer-based sequencing methods may fail to identify a significant number of proteins for such reasons as low homology of the protein of interest to related database entries, post-translational modifications, or simply that the protein is unrelated to any database entry (Kinter and Sherman, 2000). Despite these inherent drawbacks, database mining benefits usually far outweigh the costs since the aim is to find concentration changes in known proteins for future exploration.

For the most part, the relative strengths and weaknesses of MALDI versus ESI-based experiments are viewed differently by investigators. An indisputable strength of MALDI-TOF is the ease of instrument operation and the acquisition of high-sensitivity, high-resolution mass spectrum, and an indisputable weakness is the difficulty of obtaining any product ion spectra that might be needed to characterize a structure (Kinter and Sherman, 2000).

2. PURPOSE AND AIMS OF STUDY

The kidney is highly susceptible to damage from chemical and xenobiotic compounds because it receives 25% of cardiac output directly from the heart and functions as a filter by removing and concentration bodily wastes for excretion. This makes the kidney especially susceptible as a target organ for injury from uncharacterized toxin/toxin mixture exposures in theater. Greater than 40% loss of kidney function may be lost before physical symptoms indicate the need for medical interdiction and, at that level of loss, renal damage may be irreversible. If kidney decrement could be monitored in field on a routine basis by minimally or non-invasive means, the warfigher could be provided with a viable means to provide detection/protection from toxin exposures and, if exposed, treated earlier while full recovery is still an option. Currently, kidney injury can only be measured using clinical urine tests measuring nonspecific excreted protein levels and creatinine while measuring blood serum for blood urea nitrogen (BUN). These data are then combined to estimate the Glomerular Filtration Rate (GFR). The drawbacks of the use of GFR to monitor renal injury are that these tests are not fieldable, nor real-time, and nor particularly indicative of low level renal damage.

The purpose of this project is to study the differential protein levels in rats comparing normal versus animals with low-level kidney degradation in an effort to identify small, low-abundant proteins that could serve as biomarkers in a highly sensitive test for sub-clinical kidney degradation. Three well-characterized nephrotoxins will be used to target specific kidney sections in an effort to identify protein(s) useful for determining overall kidney health in a time- and dose-dependent manner. The working hypothesis of this study is that small, low-abundant proteins can be electrophoretically separated in order to compare and contrast the protein volumes of control rats versus diseased rats in order to identify protein biomarkers of low-level kidney degradation initiated by toxin exposure.

Specific Aims

(1) Enrich serum samples by removing large, over-abundant proteins (albumin, transferrins, IgG, etc.) using an immunodepletion chromatography column.

(2) Separate enriched serum based on isoelectric point and molecular weight using 2-dimensional Difference in Gel Electrophoresis (DIGE).

(3) Isolate proteins of interest from the 2D DIGE gels using analytical software.

(4) Sequence and identify protein peptides using MALDI-TOF/TOF mass spectrometry and MASCOT database.

(5) Research identified proteins for their potential as biomarkers.

3. ANIMAL STUDY DESIGN

3.1 Design of Animal Study 1 and Study 2

The rat model was chosen for this project because it is a well characterized animal model used in many toxicity studies. Additionally, rat serum can be drawn in greater volumes than from mice according to IAACUC regulations and the enrichment and 2D DIGE techniques used in this study require significant amounts of protein in order to produce reliable data. The serum samples for the BEA and Puromycin drugs came from Study 1 which was aimed at studying the low level nephrotoxicity of four drugs: BEA, d-serine, puromycin, and amphotericin B (discussed in depth in DelRaso, et al 2009). However, problems arose during the study from a bacterial contamination of samples, equipment, and subjects resulting in loss of samples in irregular numbers. Because of the contamination, only the BEA, puromycin, and d-serine studies had ample and reliable samples. However, the contamination of random samples threw off the statistical confidence of much of the produced data. Therefore the limited number of viable samples led to the need to pool samples for this study which eliminated the ability to compare across and within each nephrotoxin in a truly statistical robust approach.

D-Serine study 2 was conducted similar to Study 1 with some changes in basic study design, sample handling/preparation, and in animal handling instigated from issues learned from sample analysis from Study 1. The two major protocol changes between d-serine Study 1 versus Study 2 were the source of control samples and sample time points. In Study 1, an internal control sample came from each rat by drawing a pre-dose sample for comparison against 24 hour post dose and 96 hours post dose blood draws. After 96 hours post dose, the rats were sacrificed and this sample labeled ―Terminal Sacrifice". Though an internal control is desired and multiple samples from one rat have greater confidence of variable control, not enough serum was obtained for the multiple studies. Therefore, in Study 2 each rat was individually sacrificed at the designated time point and all the blood (serum) from the rat was collected at that time. Using this method ensured enough serum protein quantity for all the studies at hand. In addition to changing the method of sample serum draws, analysis of d-serine Study 1 data by LC/MS/MS demonstrated most changes in protein concentrations occurred between 12 and 24 hours

post dose. Therefore, instead of a serum sample taken pre-dose, 24 hours post dose and at terminal sacrifice as previously accomplished in Study 1, blood was collected at terminal sacrifices at 12 hours post dose and 24 hours post dose.

Animal/sample handling changes included randomly distributing rat subject cages among the three shelves in the housing room. Study 1 placed all the animals in groups according to their dose and kept all the same doses on the same shelves. LC/MS/MS analysis of Study 1 indicated that protein variations that could be attributed directly to shelving placement of the rat subjects by PCA analysis of urine proteomics (discussed in Shiyanov, et al. 2009). Additionally, serum and urine samples taken in Study 2 were immediately mixed with protease inhibitor cocktails to minimize degradation during collection and storage times.

3.2 Nephrotoxin Selection and Dosage

Nephrotoxins chosen for study in IACUC Protocol # F-WA-2003-0074-A were used at concentrations representative of toxicity and non-toxicity. The chemicals, dose range, method of dosing and regional toxicity in kidney are shown in Table 1. These dose ranges were supported by previous results generated under preliminary studies which were conducted in early phases of associated research projects as well as published data. For the initiation of demonstrable kidney injury, the lowest dose caused no observable abnormality in clinical chemical and histopathology, while the highest dose resulted in mild histopathological lesions and/or clinical chemistry.

Table 1: Chemical toxicants used in Phase I

Dosing Solution (15 mL)	Dose Range (~0.25 kg rat)	Dosing Method (10 mL/kg)	Regional Toxicity
Bromoethylamine *(0 – 50 mg/mL)*	0 - 500 mg/kg	*i.p.* injection	renal papillary necrosis & renal fibrosis
D-Serine *(0 – 20 mg/mL)*	0 - 200 mg/kg	*i.p.* injection	proximal tubular necrosis
Puromycin *(0 – 15 mg/mL)*	0 - 150 mg/kg	*i.p.* injection	glomerular injury

Amphotericin B *(0 – 2.5 mg/mL)*	0 - 25 mg/kg	*i.p.* injection	distal tubular damage

Vehicles used: Vegetable oil (i.e. corn, sesame, etc.) - Hydrophobic compounds
Normal saline - Hydrophilic compounds

3.3 Animal Study Protocol

3.3.1 Animal Weight and Sex Requirements

The animals were ordered at 200-225 grams. Based on the growth charts for male Fischer 344 rats available on the Charles River Laboratory website (www.criver.com) the animals, prior to chemical exposure, were allowed to reach approximately 250 grams. At this weight, the animal will obtain a circulating blood volume of 17.50-21.00 ml which is recommended for the safe removal of multiple blood samples within a 2-4 week period (NIH Office of Animal Care and Use, www.oacu.od.nih.gov). While both Study I and II utilized males, future work extending both the discovery and pre-validation of protein markers should also include a similar study utilizing female rats to determine the effect of gender difference on genomics and proteomics in response to nephrotoxicant exposures.

3.3.2 Study Procedures

Male Fischer 344 rats (200-225 gram) was obtained from Charles River Laboratories and housed in Building 838. Food and water was available for all animals *ad libitum*. On the seventh day after arrival 0.8 mL of blood, which served as the baseline, was collected from each rat via the lateral tail vein using a 23 G needle attached to a 1 cc syringe. At the end of the 14-day quarantine/acclimation period, the animals were randomly assigned to a control or one of the four treatment groups in each chemical treatment study (listed in Table 1) and housed in metabolism cages. Selection of each test chemical was based on a complete and site specific representation of the regions of the kidney, as described in the Animal Protocol # F-WA-2003-0074-A. Based on previous histopathology/clinical pathology results, the low dosages were expected to result in initiation of visible kidney injury via histopathology and clinical chemistry, while the higher dose treatment will result in mild-moderate histopathological lesions

and/or clinical chemistry. Two intermediate doses were also included. The range of Study 2 dosages for each chemical is listed in Table 1 shown above.

The schematic representation of the general experimental design is shown in Figure 6. Seventy-eight rats per chemical treatment experiment were required: (n = 3 per treatment group x 5 groups (control + dose) x 5 time points + 3 animals @ time 0). Based on the results of Animal Protocol # F-WA-2003-0074-A and that reported in the open literature, the recovery period covered a range of up to 7 days.

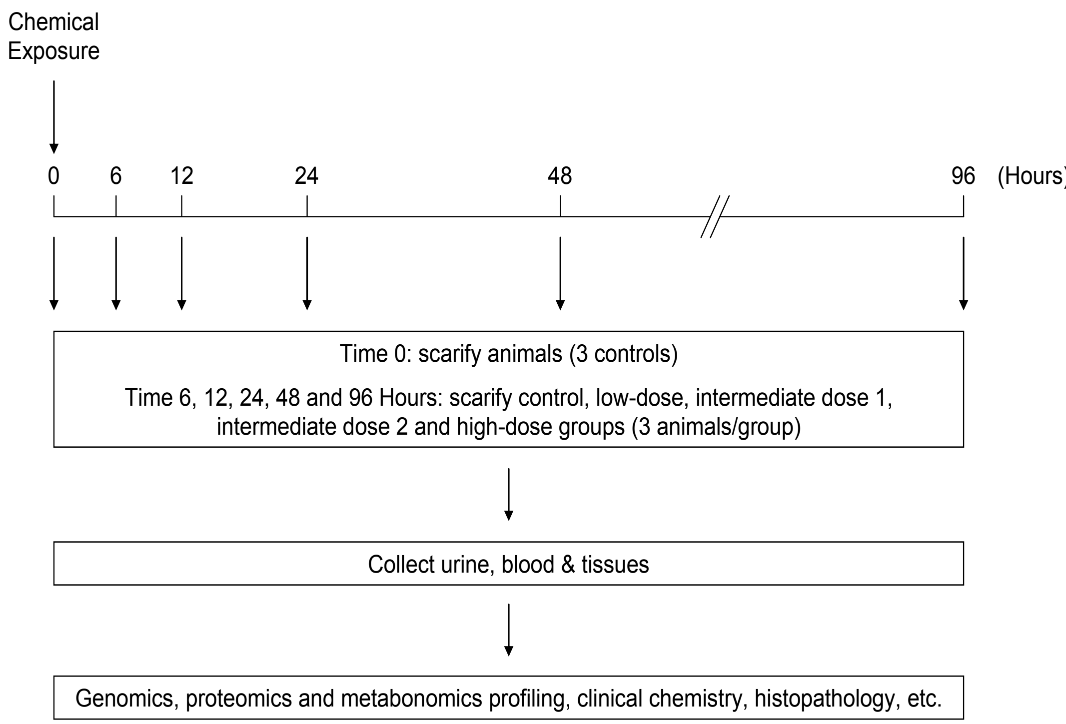


Figure 6: Schematic representation of the general design of the experiment.

On day 0 at time 0, each animal was given a single dose of either vehicle only or the test chemical at the selected doses by intraperitoneal injection at a volume of 10 mL/kg. Urine samples for proteomics and metabonomics analysis were collected 24 hours prior to dosing and daily thereafter from both control and treatment groups into 50

mL conical tubes containing 1.0 mL of 1% sodium azide maintained at 6-10°C using I-Cups (Bioanalytical Systems, Inc.).

On day 0 at time 0, three rats dosed with vehicle only were sacrificed by CO_2 asphyxiation. Blood was drawn via the inferior vena cava with an 18-20 G needle fitted to a 10 cc syringe, and the kidneys were removed for later examination. Peripheral blood mononuclear cells (PBMC) and serum/plasma was prepared from the collected blood for genomics, proteomics, metabonomics, clinical chemistry and histopathological analyses. A list of traditional renal function assays and previously described renal injury tests shown in

Table 2 were performed. Kidney tissues were frozen in liquid nitrogen for genomic profiling for later analysis. Tissues were also fixed in 10% formalin for histopathological analysis using hematoxylin and eosin staining followed by light microscopic evaluation. Since gene/protein expression can be influenced by the circadian rhythm, inclusion of the control group will help identify gene/protein expression changes unrelated to chemical exposure and thus eliminate them from the list of potential biomarkers.

At 6, 12, 24, 48 and 96 hours post-dose, 3 rats per dose group (control plus 4 dose groups) were sacrificed in the same manner. Urine, blood, and tissues were collected, processed and analyzed as at time 0. The total animal usage for the Phase I study was 312 animals (78 animals per chemical x 4 chemicals).

Table 2: Serum and Urine Analytes (Parameters) for Determination of Renal Function

Serum (or Plasma)	
Creatinine	Cystatin C
Urea	Electrolytes (sodium, calcium and magnesium)
Urine	
Volume	IgG
Creatinine	α1-microglobulin
Total protein	α2-microglobulin
Glucose	β2-microglobulin (Cleaved products)
Electrolytes (sodium, calcium and magnesium)	N-acetyl-β-D-glucosaminidase
Albumin	adenosine deaminase binding protein
α-Glutathione-S-transferase	Kidney Injury Molecule-1
π/μ-Glutathione-S-transferase	neutrophil gelatinase-associated lipocalin
Collagen IV	Clusterin
Pap X 5C10 antigen, Pap A1	IL-18
Leucocyte esterase	Lactate dehydrogenase
hemoglobin	Aspartate aminotransferase
Alanine aminopeptidase	Clara cell protein (CC16)
Leucine aminopeptidase	retinol binding protein
Neutral endopeptidase	beta 2-glycoprotein-1
gamma glutamyltransferase	Cysteine-rich protein 61, CYR61

3.3.3 Time Series Exposure Experiments

Male Fischer 344 rats (200-225 gram, from Charles River Laboratories were purchased and kept in quarantine. On the seventh day after arrival 0.8 mL of blood (serving as the baseline) were collected from each rat. At the end of the 14-day quarantine/acclimation period, the animals were randomly assigned to a control or one of the four treatment groups in each chemical study and housed in metabolism cages. Selection of the doses was guided by the results of the dose-response experiment as previously described. The low-dose treatment was expected to result in no observable abnormality in histopathology, clinical chemistry, renal function assays and metabonomic profiles while the high-dose treatment is expected to result in mild nephrotoxicity. Two intermediate doses were also included.

Seventy-eight rats per chemical treatment experiment were required (n = 3/treatment group; vehicle control and 4 dose groups). Based on previous results of dose-

response experiments, as well as those reported in the open literature, experimental exposure and recovery period was expected to extend up to 14 days.

On day 0 at time 0, each animal was given a single dose of either vehicle only or the test chemical at the selected doses by intraperitoneal injection (or oral gavage) at a volume of 10 mL/kg. Urine samples, for proteomics and metabonomics analysis, were collected 24 hour prior to dosing and daily thereafter from both control and treatment groups. The urine was corrected from cage directly into 50 mL conical tubes containing 1.0 mL of 1% sodium azide maintained at 6-10°C using I-Cups (Bioanalytical Systems, Inc.).

On day 0 at time 0, 3 rats dosed with vehicle only were sacrificed by CO_2 asphyxiation. Blood was drawn via the inferior vena cava with an 18-20 G needle fitted to a 10 cc syringe, and kidney removed. Peripheral blood mononuclear cells (PBMC) and serum/plasma were prepared from the collected blood for genomics, proteomics, metabonomics, clinical chemistry and histopathological analyses. Kidney tissues were frozen in liquid nitrogen for genomics profiling or fixed in 10% formalin for histopathological analysis. Tissue samples were later stained with hematoxylin and eosin for light microscopic evaluation to verify injury.

At 6, 12, 24, 48 and 96 hours post-dose, 4 rats per dose group (control plus 4 dose groups) were sacrificed in the same manner. Urine, blood and tissues were collected, processed and analyzed as at time 0. The total animal usage for the Study I was 780 (10 chemicals x 5 dose groups x 5 time points x 3 animals/time point) + (10 chemicals x 1 dose group (control) x 1 time point (time 0) x 3 animals/time point).

3.3.4 Clinical Chemistry, Renal Function Test and Histopathology Analysis

Blood samples were collected in non-additive red top clot tubes (Becton-Dickinson) and the serum recovered by centrifugation at 2,500 x g at 25^{o}C for 10 min. Serum chemistry for specific analytes such as creatinine, urea, sodium, calcium and magnesium were analyzed using a Roche Cobas Mira Clinical Chemistry Analyzer. As

part of an associated study examining the same samples (Mauzy, et al. 2009) urinary creatinine, total protein, glucose, sodium, calcium, magnesium albumin hemoglobin, IgG, α1-microglobin, α2-microglobin, β2-microglobin, lactate dehydrogenase, aspartate aminotransferases, α and β glutamyltransferases, and alanine aminotransferases were also determined. Other urinary proteins associated with renal injury such as N-acetyl-β-D-glucosamindase, adenosine deaminase binding protein, α-glutathione-S transferase, μ-glutathione-S transferase, neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, clusterin, retinol binding protein, etc. were determined using either Western blots or ELISA (see Table 2 for the entire list of serum and urinary analytes) and also reported in Mauzy et al. Tissue samples were collected for histopathology and placed in 10% neutral-buffered formalin followed by sectioning (1-3 mm), paraffin embedding, cutting, placement on slides, and staining using hematoxylin and eosin.

4. EVALUATION OF MARS COLUMN

4.1 Methods

4.1.1 Serum Enrichment using the MARS column

The first attempt at removing high-abundant proteins from the rat serum samples used an HPLC version of the Multiple Affinity Removal System for Mouse Serum Proteins (MARS MS-3) column purchased from Agilent. This column utilizes antibody-antigen interactions to capture high-abundant proteins and remove them from the sample serum. As previously discussed, the removal of the high-abundant proteins is required to optimize the visualization and study of low-abundant proteins with proteomic analysis techniques such as 1D and 2D gel electrophoresis and mass spectrometry.

To begin the serum immunodepletion, Agilent‘s proprietary Buffer A and Buffer B were poured into the HPLC reservoirs and the HPLC lines were purged, first with Buffer A then with Buffer B, at a flow rate of 1.0 mL/min for 10 min without a column to ensure all lines were flushed of any mobile phase from previous use. Afterwards, the MARS column was installed in the HPLC and the column was conditioned using Buffer A. Next, the LC timetable was set up and two method blanks were run by injecting 200 µL of Buffer A without a column. The column was then attached and equilibrated for 4 min with Buffer A with a flow rate of 1 mL/min at room temperature.

Once the HPLC lines and MARS column were flushed and equilibrated, rat serum sample 119, a BEA control subject from study 1; sample 245, a control from D-Serine study 1; and sample 305, a puromycin control subject from study1, were diluted 1:5 by combining 90-µL of serum with 360-µL of Buffer A. The diluted samples were filtered using a 0.22-µm spin filter for 1 min at 16,000 x g. Next, 450 µL of each diluted serum was injected into the HPLC and run through the column at a flow rate of 0.5 mL/min using 100% Buffer A for 10 minutes. The Flow-Through (FT) fraction was collected by autosampler from 4 to 6 min in one minute intervals, giving 0.5 mL volumes then stored for later analysis. The bound proteins were eluted with 100% Buffer B at a flow rate of 1 mL/min for 7.0 min. The autosampler was programmed to collect the Elution (E)

fractions from 11 min to 15 min collecting 1 ml per minute. Once the bound proteins were eluted, the column was regenerated by running 100% Buffer A for 11.0 min at a flow rate of 1 mL/min. Once the FT and E fractions were collected, they were concentrated and the buffer exchanged for 2M urea. This concentration/buffer exchange was done using an Amicon YM-5, a 5 kDa molecular weight cutoff spin tube in a standard 15 mL plastic collection tube. The HPLC protocol is depicted with a sample chromatogram from the Agilent manual in (Figure 7).

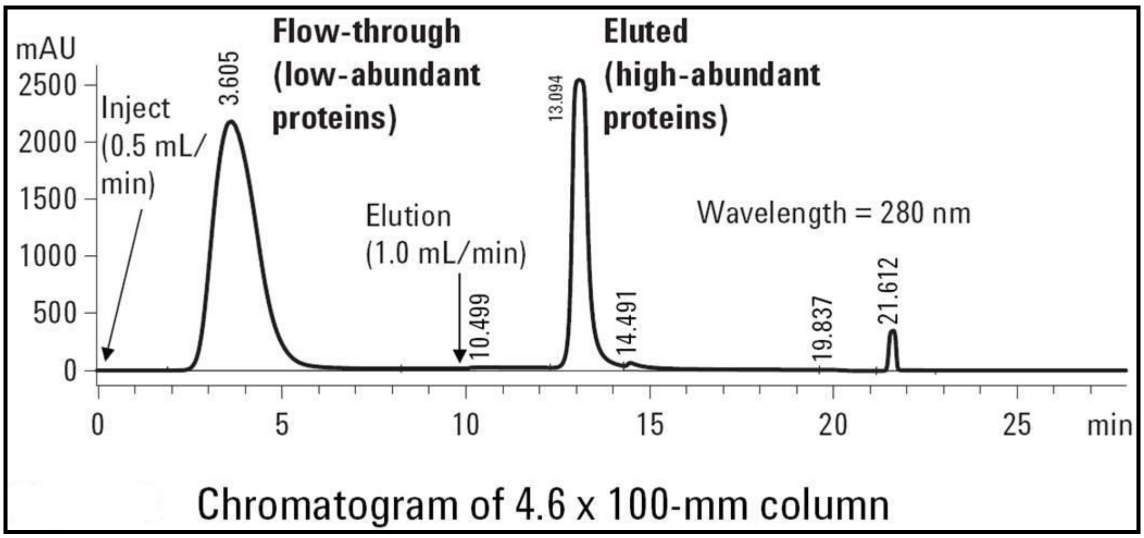


Figure 7: Sample chromatogram from Agilent Protocol

4.1.2 Protein Assay

In order to run 2D DIGE gels, the exact protein concentration is needed in each sample as equal amounts of protein mass (in µg) are required for the imaging statistics to work properly. Additionally, regular 2D PAGE gels with silver staining require known protein concentrations so that the gels are not overloaded, which decreases gel resolution and protein identification. For this study, the protein assay also served as a quality control measure since the amount of protein in the FT and E fraction of each sample can be compared and used to indicate column removal efficiency.

The protein assay used most often was the Non-Interfering (NI) Protein Assay kit from GBiosciences. First, the kit was laid out along with a 2 mL 96 well plate (Fisher

#07-200-701) and the serum samples sitting in ice. A standard curve was set up by adding 25, 20, 15, 12.5, 8, 5, 4, 2, 1, and 0 µL of BSA (2 µg/µL) in triplicate to the plate followed by serum samples which were added in 2 µL quantities in triplicate to the plate. Next, 500 µL of UPPA-1 was added to each well using a repeater pipet and 10 mL plastic syringe. Adhesive film (Fisher #05500-32) was placed over the plate and the entire plate was vortexed for approximately 30 seconds. The plastic film was removed and the samples allowed to incubate at room temperature for 2 -3 minutes. Next, 500 µL of UPPA-II was added to each well, a plastic adhesive film placed over top and vortexed briefly. The plate was weighed and then, a balancer plate was weighed and adjusted to within 0.05 g of the sample plate using water. The plates were centrifuged on a Dupont Sorvall RT6000D using 11093 rotor at 3400 x g and 4^{o} Celsius for 15 minutes. While centrifuging, Reagent II was mixed by adding 100 parts Color Reagent A with 1 part Color Reagent B with enough for 1 mL per sample. After centrifuging, the supernatant was discarded by inverting the plate in the sink and blotting off excess liquid on paper wipes. Next, 500 µL of UPPA-1 was added to each well, taking care to not disturb the pellet at the bottom. Then 100 µL of UPPA-II was added to each well, the plate covered with plastic adhesive film and gently inverted – not vortexed - a few times. The plate and a balancer plate were again weighed and adjusted until balanced and centrifuged as before but for only 10 minutes. Afterwards, the supernatant was poured off and excess liquid blotted off with labwipes. Then, 100 µL of Copper Solution (Reagent I) was added to each well and followed by 400 µL of nanopure water in each well. The plate was covered with plastic adhesive film and vortexed to dissolve the pellet. Next, 1 mL of Reagent II was added to each well and the samples allowed to incubate for 15 – 20 minutes at room temperature. Aftewards, 200 µL of each solution was transferred to a microtest 96 well plate (Fisher #12-565-501) using a 12 channel pipet. Lastly, 200 µL of nanopure water was added to three wells for blanks. The plate was read using a Molecular Devices SpectraMax 190 at 480 nm and the data collected using Softmax Pro software. All data was processed using Excel.

4.1.3 2-Dimensional Polyacrylamide Gel Electrophoresis (2D PAGE)

Separation in the 1st Dimension

Isoelectric Point Gradient (IPG) strips were prepared to separate the serum proteins by isoelectric point in a pH gradient ranging from 4 to 7. First, the sample was pipetted into the lanes of a re-swelling tray. Next, the plastic liners were removed from the IPG strips and the strips placed into the re-swelling tray with the plastic backing facing down. Mineral oil was added on top of all the IPG strips to prevent them from drying out, the cover was placed on the re-swelling tray to protect the sample, and the IPG strips were allowed to incubate overnight to absorb all the proteins. The next day the IPG strips were carefully removed from the trays using tweezers, excess mineral oil allowed to drip off onto labwipes, and the strips placed into a liner tray. Two electrode strips were cut to fit across the liner tray and 1 mL of water was pipetted onto each side of the electrode strip with excess water blotted off using labwipes. The electrode strips were placed across the IPG strips at the top and bottom then the plastic electrodes clamped in place. The electrode/IPG tray conglomerate was clamped in place on the electrophoresis apparatus and mineral oil was used to fill any remaining gaps. The apparatus and its cooling system were turned on, set to 300V, and allowed to run overnight in an 18 hour gradient cycle reaching a max of 3000V.

As the serum separated in the 1st dimension, the gel tank and necessary buffers were prepared for running the gel in the 2nd dimension the next day. Previously mixed stock buffer solutions in 10X concentrated form were prepared and kept in the cold room. The lower tank buffer was prepared by pouring 8 L of Millipore water into the lower tank and mixed with stock buffer, which was diluted by mixing 1 L of 10X concentrate with 1 L of Millipore water in a 2 L graduated cylinder. The diluted solution was left in the tank overnight with the cooling fan turned on and the tank covered overnight. The Upper buffer was prepared by diluting 300 mL of 10X Upper buffer solution to 3 L in a 4 L Erlenmeyer flask with Millipore water. The diluted solution was transferred to a 3 L plastic beaker and placed on a stir plate with stir bar and covered until the next day.

Separation in 2^{nd} Dimension - Protocol

The following day the samples and apparatus were ready for separating the serum proteins in the 2^{nd} dimension. First, two 50 mL conical tubes of equilibration buffer were taken from the cold room and allowed to thaw. To one tube was added 800 mg of dithiothreitol (DTT) and to the other tube was added 1 g of iodoacetamide (IAA). The IAA tube was wrapped in aluminum foil since IAA is light sensitive. Both conical tubes were placed on a rocker plate to warm and dissolve the newly added chemicals. Once thoroughly mixed, the equilibration buffer/DTT and equilibration buffer/IAA solutions were filtered in their own disposable 0.22 micron vacuum filter beakers (covering the IAA beaker in aluminum foil again) to remove any particulates. In the mean time, the IPG strips were removed from the electrode tray using tweezers and labwipes to blot off excess mineral oil. The strips were placed into a re-swelling tray, covered with the DTT buffer, wrapped in aluminum, and placed on a rotating shaker (BellyDancer ® shaker, ATR Co.) for 15 minutes. After the 15 minutes, the DTT buffer was vacuumed up and the process repeated three more times, once more with DTT and twice with the IAA solution giving a total of four washes in equilibration buffers. Next, the gel apparatus was prepared by placing spacers into the slots in the upper tank and double paned, pre-cast gels purchased from Nextgen Sciences Inc (Cat # PRS-703696) were placed into the tanks as well. Then, the Upper buffer was poured into the upper tank until the tops of the gels were barely covered, the IPG strips placed into the gel wells using tweezers, and the remaining buffer poured into the tank. The apparatus was enclosed and the gel ran at 500 mA for 5 hours, 51 minutes. Once the proteins had separated, the gels were removed from the apparatus, cut from the glass plates, silver stained according to protocol, then digitally scanned for analysis.

Proteosilver Silver Stain

Sigma‘s ProteoSilver™ Silver Stain Kit was used to silver stain the small, precast gels purchased from Invitrogen. The final volume for each solution mixed for this kit depended on the number of gels to be stained since each gel required 200 ml of each solution. First, the gels were placed into a clean tray (up to 2 gels per tray) to soak in Fix 1 solution (50% Ethanol, 10% Acetic Acid) from 1 hour to overnight. The gels were

removed from the Fix 1 and placed into Fix 2 (30% Ethanol) for 10 minutes and then the gels were washed with Millipore water for 10 minutes. Afterwards, the gels were placed into Sensitizer solution for 10 minutes which was made right before use by diluting ProteoSilver Sensitizer 1:100 with Millipore water. After soaking in Sensitizer, the gels were washed with Millipore water twice for 10 minutes apiece. Next, the gels were placed into Silver solution for 10 minutes. The Silver solution was prepared by diluting the ProteoSilver Silver Solution from the kit 1:100 with Millipore water and was used immediately since the diluted solution only had an active life of 2 hours. The gels were then removed and washed for 1 minute using Millipore water. Once cleaned, the gels were placed in developer solution for 3 to 7 minutes depending on the amount of protein in the gel and the intensity of the staining desired. The Developer solution was made immediately prior to use by adding 5 ml of ProteoSilver Developer 1 chemicals and 0.1 ml of ProteoSilver Developer 2 chemicals to 95 ml of Millipore water. Like the other solutions, the developer was scaled up to give a final volume so that each gel would be immersed in 200 ml of solution. Once the protein spots were dark enough, but before the background became too elevated, 5 ml of the Stop solution (included in the kit) was added directly to the Developer Solution and allowed to incubate for 5 minutes.

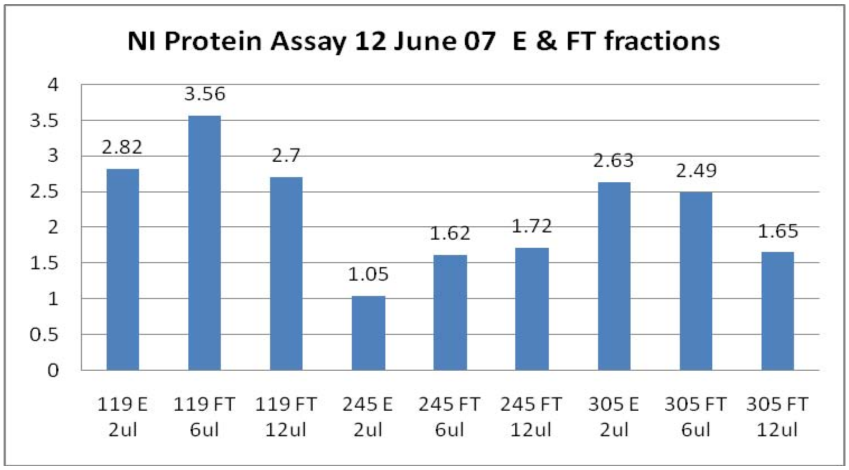
4.2. Results of MARS MS-3 Column Evaluation

Protein Assays and 2D PAGE

After serum enrichment, samples were tested by protein assays and 2D gels to gauge the performance of the Agilent column. All initial tests showed that the Agilent MS-3 column did not remove enough high abundant proteins to allow visualization of low abundant proteins. The Agilent literature states that the MARS MS-3 column is capable of removing 80% of the albumin, transferrin, and IgG from rat serum. Literature searches online and from Agilent point out that approximately 98% of serum proteins are the over abundant proteins, of which albumin, transferrin, and IgG make up the majority. Assuming that 80% of the top three abundant proteins are removed by the Agilent column, a rough calculation indicated that 78.4% of all proteins should be removed from the study serum samples. The NI protein assay measured a protein concentration of 2.82

μg/μl in the Elution fraction of sample 119 but 3.56 μg/μl in the Flow Through fraction while the assay from a week later measured a 39.38 μg/μL concentration in the whole serum of sample 119 (Figure 8).

A



B

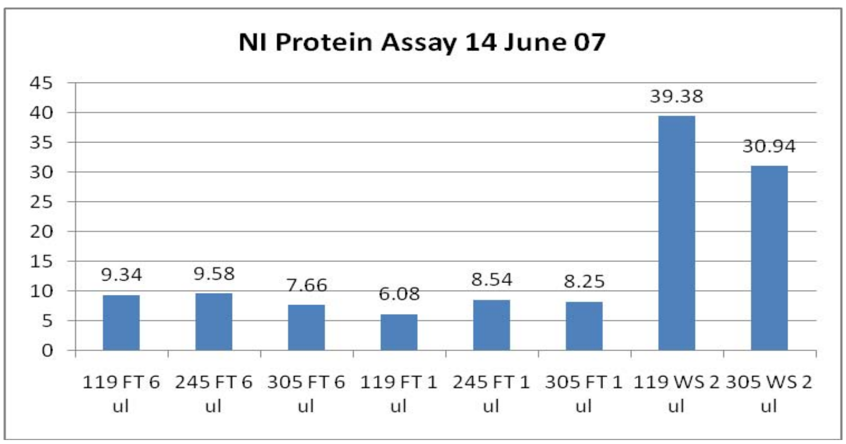


Figure 8: A) Non- Interfering (NI) Protein Assay showing protein concentrations for E and FT fractions of Control Samples 119, 245, 305 before concentration & buffer exchange. B) NI Protein Assay showing protein concentrations for E (eluate) and FT (flow through) fractions and WS (whole serum) of Control Samples 119, 245, 305 after concentration & buffer exchange.

These values mean that 7.16% of the albumin, transferrin, and IgG were captured in the E fraction and 9.04% of the proteins washed through in the FT fraction. Given the abundance of the top three, one would have expected to see a greater concentration of proteins in the E fraction compared to the FT fraction. These values also indicated that a large majority of the proteins were being lost in the concentration/buffer exchange phase

since one expects a greater protein recovery than 16.2% combined. The evidence that the Agilent column was not effective was further demonstrated in the large 2D, silver stained gels shown below (Figure 9A, 9B).

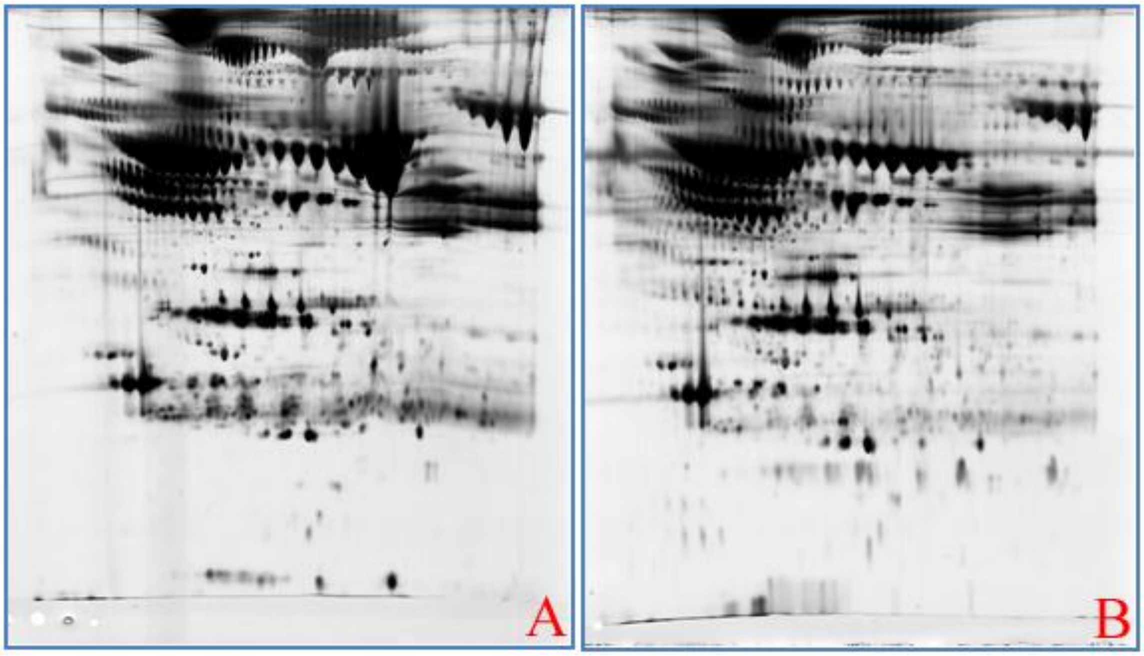


Figure 9: **A) 2D PAGE of Sample 119 Whole Serum B) 2D PAGE of Sample 119 Flow Through after immunodepletion with Agilent MARS MS-3 HPLC**

Comparison between the FT fraction gel and the Whole Serum gel shown in Figure 9A and 9B demonstrate that very little enrichment occurred using the Agilent MARS column. Similar tests using 305 (Figure 10A, 10B) examining FT after one pass through the MARS MS-3 column indicate that this is also not a sample specific issue. The large swaths of proteins and low spot resolution indicate that immunodepletion required additional optimization in order to achieve relevant sample cleanup for further analysis.

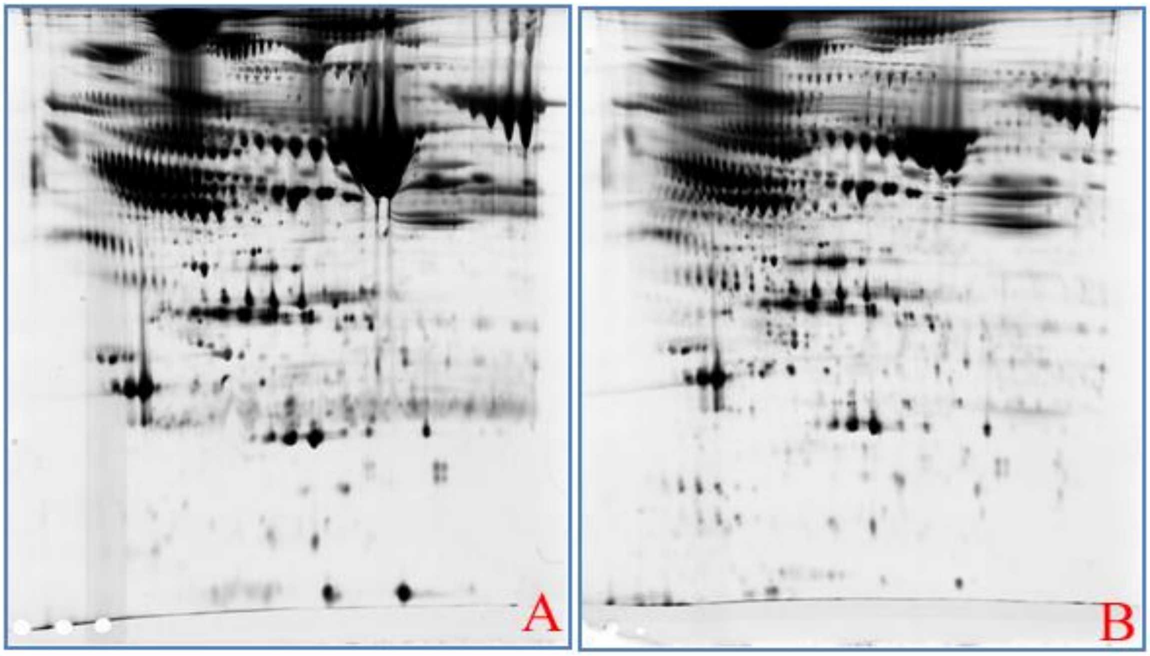


Figure 10: A) 2D PAGE of Sample 305 Whole Serum B) 2D PAGE of Sample 305 Flow Through after immunodepletion with Agilent MARS MS-3 HPLC

4.3 Discussion of MARS Evaluation Results

Based on the evidence from the protein assays and gel images, it was concluded that the Agilent HPLC MARS column did not work as advertised and optimization tests were needed. The large swaths of over abundant proteins prevent any ability to visualize smaller, low abundant proteins by 2D DIGE gel and would also prevent any image analysis. Optimization tests included reducing the amount of rat serum injected into the column and running the column at slower flow rates. Additionally, the centrifuge version of the MARS column was tested and both HPLC and spin columns were tested with commercially purchased mouse serum. The mouse serum was used as a control since the MARS column was advertised specifically for mouse serum. Therefore, the data indicate that the Agilent MARS column did not work as well as described by Agilent even using the optimal sample matrix (mouse).

5. MARS COLUMN OPTIMIZATION

5.1 Examination of Serum Dilution

5.1.1 Methods for Analyzing Serum Dilution Effects

To test the theory that the MARS column was potentially overloaded with too much serum proteins, sequentially less volumes of serum were diluted 1: 5 per the protocol and injected into the column. The spectrometry data was coupled with 1D SDS-PAGE gels to identify an optimal volume that produced better separation of proteins. The test used 5 volumes (10, 20, 30, 40, 50 µL) all of which were diluted 1:5 and run through the HPLC column according to Agilent‘s protocol as detailed previously.

5.1.2 Results of Serum Dilution Analysis

The consistent band sizes and intensities across all samples in both gels demonstrate that there is no difference in MARS column effectiveness as the amount of total protein is decreased in the sample (Figure 11, Figure 12). This fact meant that either this specific MS-3 column was defective due to either manufacturing or shipping problems or simply that rat proteins do not bind well to the Agilent column.

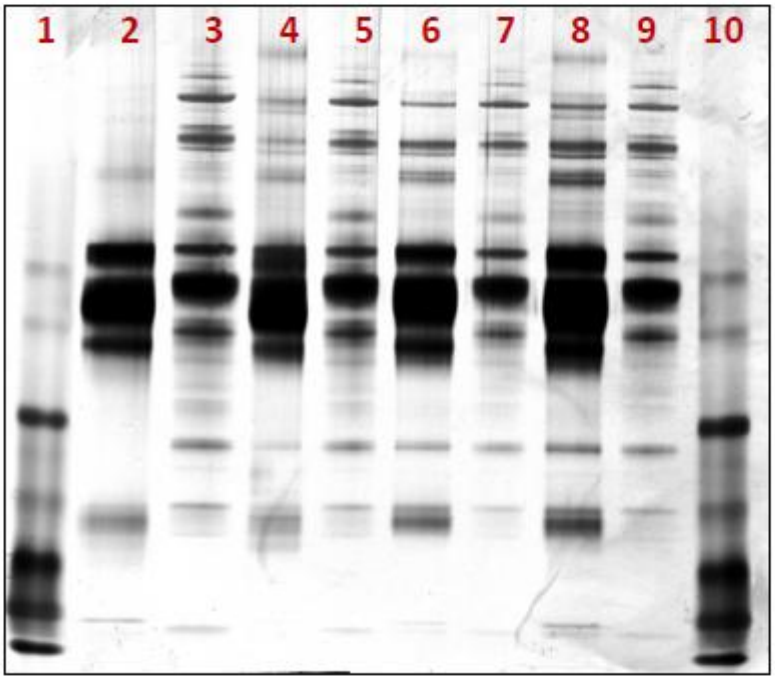


Figure 11: 1D SDS-PAGE of collected FT and E fractions of rat Control sample 119 from MARS HPLC column immunodepletion with sample volumes ranging from 10–40 ul.

The samples are in lanes 2 – 9 and are as follows:

1) *Blue MW Standard*
2) *E fraction of 10 ul of sample 119*
3) *FT fraction of 10 ul of sample 119*
4) *E fraction of 20 ul of sample 119*
5) *FT fraction of 20 ul of sample 119*
6) *E fraction of 30 ul of sample 119*
7) *FT fraction of 30 ul of sample 119*
8) *E fraction of 40 ul of sample 119*
9) *FT fraction of 40 ul of sample 119*
10) *Blue MW Standard*

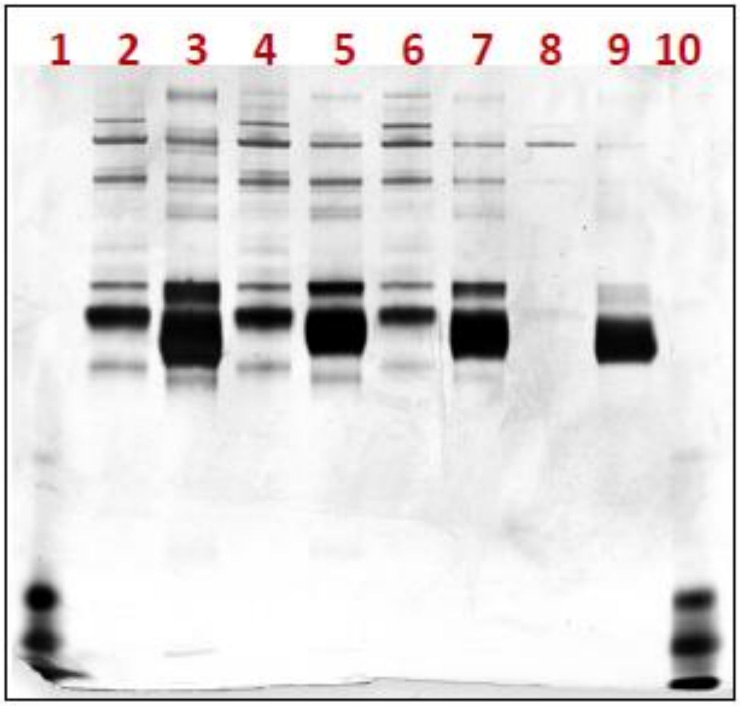


Figure 12: 1D SDS-PAGE of collected FT and E fractions of rat Control sample 305 after MARS immunodepletion at 10 – 40 ul.

The samples are in lanes 2 – 9 and are as follows:

1) *BlueMW Standard*
2) *FT fraction of 10 ul of sample 305*
3) *E fraction of 10 ul of sample305*
4) *FT fraction of 20 ul of sample 305*
5) *E fraction of 20 ul of sample 305*
6) *FT fraction of 30 ul of sample 305*
7) *E fraction of 30 ul of sample 305*
8) *FT fraction of 40 ul of sample 305*
9) *E fraction of 40 ul of sample 305*
10) *Blue MW Standard*

5.2 Testing MARS Flow Rate

5.2.1 Method for Examining Flow Rate Effects

To test the theory that the overabundant proteins would bind to the column matrix with greater efficiency if given more time, the flow rate was reduced from 0.5 ml/min to 0.25 ml/min. The general protocol from Agilent was otherwise unchanged and followed the outline described previously.

5.2.2 Results of Flow Rate Analyses

Figure 13 shows the FT and E fractions after changing the HPLC run to collect more of the FT fraction from sample 119 and 305 in gel lanes 2 – 8. This change in protocol is denoted by 01A at the end of the sample name. One unexpected result was the collection of a second elution peak for sample 305 run under the 01A protocol. This fraction was tested in Lane 8 to see if there were any significant differences from the other fractions. Lanes 9 and 10 show the FT and E fractions of sample 305 after the protocol was changed to a slower flow rate of 0.25 ml/min from the protocol 0.5 ml/min to give the top three proteins longer time to bind to the mouse-3 column. This change in protocol is denoted by 02 at the end of the sample name. The consistent protein bands across all FT and E fractions in the gel show that no binding improvement was achieved. Based on this result, it was inferred that rat protein homology differs from mouse proteins in ways that prevent the stationary phase from efficiently binding and removing over-abundant proteins.

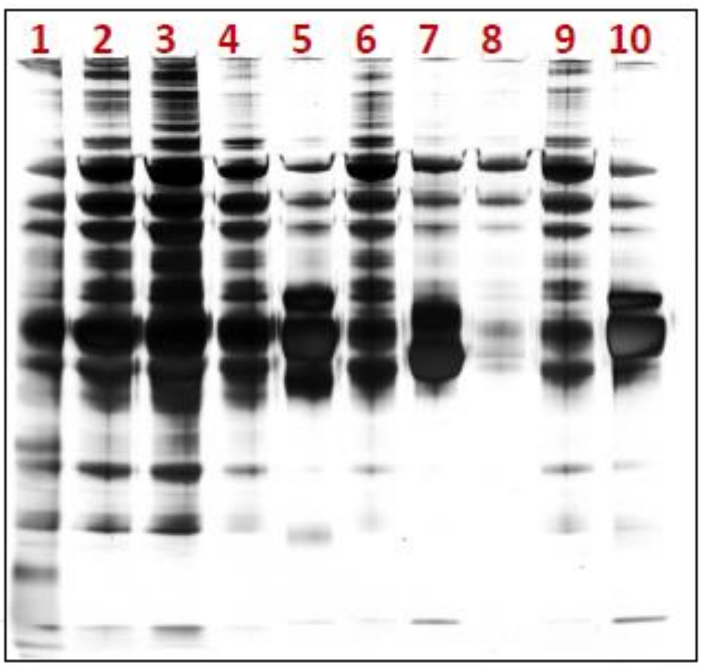


Figure 13: 1D SDS-PAGE of Samples 119 and 305 using Agilent HPLC MARS MS-3 column after change in protocol to test enrichment effects of slower flow rate.

The samples are are as follows:
1) Blue MW Standard
2) FT fraction 20 ul of sample 119 01A run 1
3) E fraction 20 ul of sample 119 01A run 1
4) FT fraction 20 ul of sample 119 01A run 2
5) E fraction 20 ul of sample 119 01A run 2
7) E 1 fraction of 20 ul of sample 305 01A
8) E 2 fraction of 20 ul of sample 305 01A
9) FT fraction of 20 ul of sample 305 02
10) E fraction of 20 ul of sample 305 02

5.3 Positive and Negative Control Tests for Column Matrix Animal Specificity

5.3.1 Control Test Methodology

The MARS MS-3 column was designed to remove the top three over-abundant proteins from mouse serum but this study was conducted in a rat model. As rat proteins have a different morphology from those found in the mouse, the MARS HPLC column was tested using commercially purchased mouse serum. The Agilent HPLC protocol was strictly followed as described previously. After testing the *HPLC* MARS MS-3 column, a MARS MS-3 *spin* column was tested to discern if the HPLC column was manufactured incorrectly or possibly damaged during shipping. Both commercially purchased mouse serum and Control samples numbers 119 and 305 from the study were used on the spin column using the published Agilent protocol.

5.3.2 Results of Examination of Control Samples

In Figure 14, lanes 2 – 5, show collected FT and E fractions of mouse control serum purchased from Sigma. Samples of control mouse serum were run on the HPLC column by injecting 90 ul and following Agilent protocols. Once fractions were collected, a 1 ul sample and a 10 ul sample of each fraction was prepared according to protocols and run on a 1 D gel since the protein concentration was unconfirmed. Lanes 6 – 9 show the mouse control serum FT and E fractions after filtration using the Agilent Mouse-3 spin cartridge (Figure 14). Once the FT and E fractions were collected, a 1 ul sample and a 10 ul sample were prepared using standard protocols and run on a 1 D gel since protein concentration was unconfirmed.

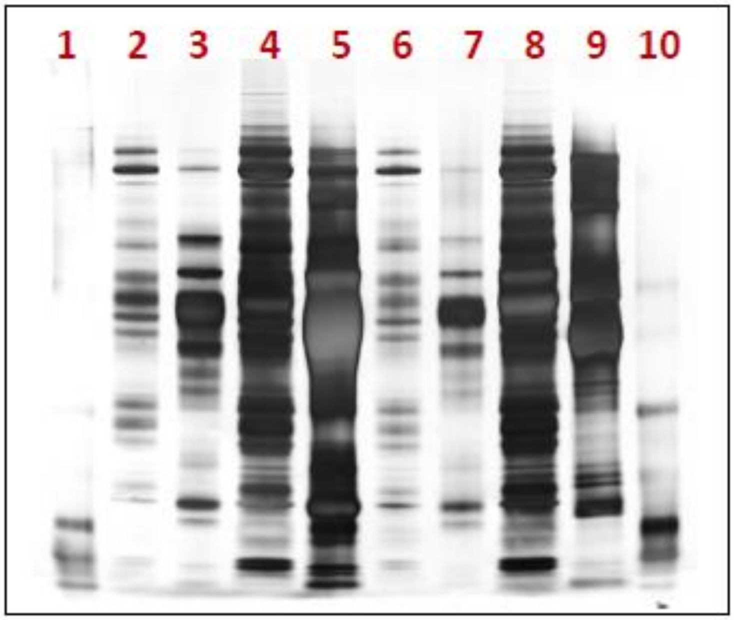


Figure 14: 1D SDS-PAGE comparing Agilent MARS MS-3 HPLC and Spin columns using commercially purchased mouse serum enriched according to protocols.

1) *Blue MW Standard*
2) *FT mouse control HPLC 1 ul*
3) *E mouse control HPLC 1ul*
4) *FT mouse control HPLC 10 ul*
5) *E mouse control HPLC 10 ul*
6) *FT mouse control Spin Column 1ul*
7) *E mouse control Spin Column 1 ul*
8) *FT mouse control Spin Column 10 ul*
9) *E mouse control Spin Column 10 ul*
10) *Blue MW Standard*

The distinct difference in protein band intensity between the Mouse FT and E fractions compared to the consistent protein band intensities of the Rat FT and E fractions (Figure 15) indicate that the MARS MS-3 column does work well at enriching mouse serum samples but does not work as advertised on rat samples

The stationary phase of the MS-3 column is simply unable to adapt to the different protein morphologies and cannot enrich rat serum for use in this project. The corroboration with the spin column confirmed that the HPLC column was not damaged and merely cannot cope with rat serum.

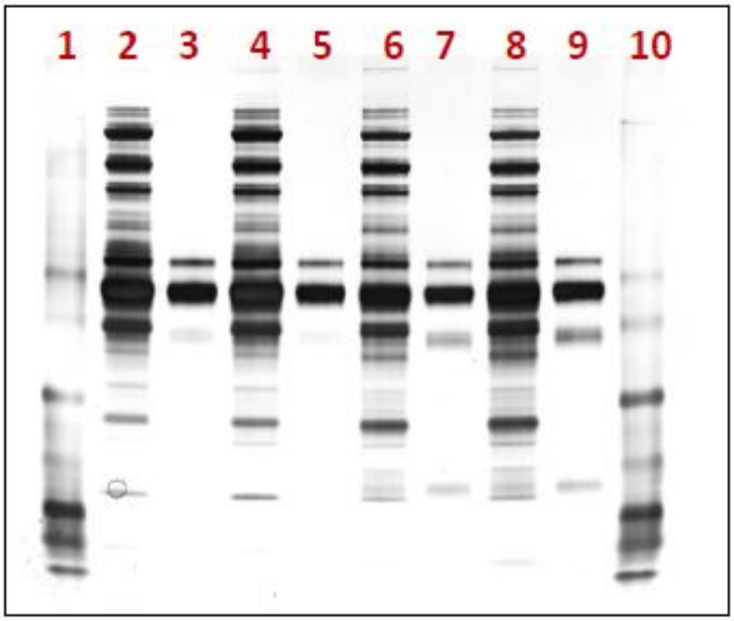


Figure 15: Negative control rat samples from puromycin (animal #305) and bromoethylamine (BEA) (animal #119) nephrotoxicity studies.

The samples were filtered through the Agilent mouse-3 spin cartridge in order to compare the depletion results with the HPLC column. The samples were run in duplicate according to Agilent protocol and separated by 1 D gel. The lanes are as follows:

1) *Blue MW Standard*
2) *FT rat sample 305 run 1*
3) *E rat sample 305 run 1*
4) *FT rat sample 305 run 2*
5) *E rat sample 305 run 2*
6) *FT rat sample 119 run 1*
7) *E rat sample 119 run 1*
8) *FT rat sample 119 run 2*
9) *E rat sample 119 run 2*
10) *Blue MW Standard*

5.4 Discussion of MARS Optimization

All of the optimization tests demonstrated that the Agilent MARS column was performing at maximum effectiveness but was still unable to provide the resolution needed for a DIGE study. Injecting smaller sample volumes to decrease the amount of protein enriched by the column demonstrated no improvement, indicating that the column was not being overloaded. Slowing the flow rate to increase antigen-antibody interaction time did not increase enrichment resolution. The final column analysis compared rat serum with mouse serum on both in the HPLC column and the spin column and confirmed that the HPLC column was not a faulty anomaly but that the MS-3 stationary

phase was simply unable to bind rat protein as effectively as described by Agilent in their column protocol.

In order to advance the project, a new column was obtained that was advertised as being effective at removing the top seven abundant proteins in rodents. The column was the IgY-R7 (chicken antibody) spin column from Proteome Labs. This column was purchased from Beckman-Coulter and field tested using commercially purchased rat serum from Sigma-Aldrich.

6. PROTEOMET LAB IgY-R7 COLUMN TESTING

6.1 IgY-R7 Immunodeletion Methods

6.1.1 Enrichment Method using the IgY-R7 Column

A demonstration IgY column kit was obtained and tested for effectiveness using 1D Gels and BCA and Non-Interfering protein assays. Commercially available rat serum was purchased from Sigma Aldrich for the IgY tests so as not to waste study samples. The rat serum was run through the column according to the Proteome Lab protocol and concentrated using Microcon YM-10 and YM-3 spin cartridges.

Use of the ProteomeLab IgY-R7 Spin Column Proteome Partitioning Kit strictly followed the protocol included with the kit. To begin using a new column, the bottom snap was removed and the column was placed into a 2 mL collection tube and centrifuged for 30 sec at 2,000 rpm to remove the storage buffer. Next, the column was conditioned by performing two complete buffer runs without adding any sample.
A conditioning buffer run began by adding 500 μL of 1X Dilution Buffer to the column, inserting the top cap, inverting and shaking the column several times to fully immerse all the IgY beads, and placing the column in a 2 mL centrifuge collection tube. The column and collection tube were then centrifuged for 30 sec at 2,000 rpm. The collection tube was removed, end cap placed on the column, and the previous step repeated a total of four times. The next step to condition the IgY beads used 500 μL of 1X Stripping Buffer and repeated the previous steps a total of four times. The final step to condition the column was to regenerate the IgY beads using 600 μL of Neutralization Buffer and repeating the previous steps a total of one time. All three steps (wash, elution, neutralization) constituted one conditioning run. The entire process was repeated a second time. The column only needed to be conditioned before its first use, no conditioning was necessary between uses on samples.

Before immunodepletion, 5 mL of 10X Dilution Buffer was mixed with 100 μL of Protease Inhibitor and diluted to 50 mL in a conical tube. The protease inhibitor was to help reduce protein degradation during the enrichment process. To begin sample

immunodepletion, 15 μL of rat serum was diluted in 485 μL of Dilution Buffer and pipetted into the spin column. The column was sealed with the top cap, inverted and shaken, then incubated at room temperature for 15 minutes. The end cap was removed from the bottom of the column and the column was placed into a 2 mL collection tube and centrifuged for 30 sec at 2,000 rpm using a desktop mini-spin centrifuge. The collection tube with the Flow Through (FT) fraction was set aside in ice and the end cap placed on the column. Next, 500 μL of Dilution Buffer was added and the beads and buffer mixed by inverting and shaking the column. Then, the end cap was removed, column placed into a 2 mL collection tube, and centrifuged for 30 sec at 2,000 rpm. The second and final FT fraction was collected and placed on ice. The same process was repeated three additional times with 500 μL of 1X Dilution Buffer to give the Wash fractions.

After washing and collecting the low-abundant proteins, the bound proteins were eluted and collected. To remove the bound proteins, the end cap was placed on the bottom, 500 μL of 1X Stripping Buffer was added to the column, and the top cap was snapped into place. The beads were mixed by inverting and shaking the column then incubated at room temperature for 2 – 3 minutes. The end cap was removed and the column placed into a 2 mL collection tube. The column and tube were centrifuged for 30 sec at 2,000 rpm. This process was repeated three more times for a total of 4 Elution fractions (E) each approximately 500 μL in volume with each fraction being stored on ice during the interim. The column should not be exposed to Stripping Buffer longer than 15 minutes to ensure column stability. Immediately after the final Elution fraction, the end cap was attached, 600 μL of Neutralization Buffer was added, and the top cap snapped on so the entire column could be inverted and shaken to mix the beads. The column was left to incubate at room temperature for 5 minutes then the end cap removed and the column placed into a 2 mL collection tube. The column and tube were centrifuged for 30 sec at 2,000 rpm. If another sample was ready it would be added to the column and the entire process repeated. If no other sample were ready, 500 μL of 1X Dilution Buffer would be added with the caps in place and the beads mixed for storage until next use.

After all nine fractions were collected and stored on ice, the two FT fractions, three W fractions, and 4 E fractions were pooled according to their respective groups into one 2 mL collection tube per group. The E fraction also included 220 µL of Neutralization Buffer to prevent protein degradation. After all samples were prepared for storage, they were labeled and placed in a -20^{o} Celsius freezer.

6.1.2 Gel Analysis of IgY-R7 Fractions

For the 1D gel test, 15 ul of commercially available rat serum was diluted with 485 ul of IgY dilution buffer for a 1:33 dilution. The diluted sample was run through the column and the first generation FT fraction was collected (1 ml) and labeled Run 3. The column was then washed and abundant proteins were stripped off and collected to give the first generation Elution fraction (2 ml). Once the column was stable, 500 ul of the first generation FT fraction was run through the column as Run 4. The Run 4 FT fraction was collected (1 ml) and the bound proteins were stripped to give a second generation E fraction (2 ml) labeled Run 4 Elution. A BCA protein assay was next in an effort to quantify the protein concentrations in each fraction. Unfortunately, the concentrations were so small they were below the sensitivity of the assay. So, the fractions were concentrated by centrifugation using a Microcon YM-3 concentration tube. This tube uses a filter and centrifugal force to collect anything greater than 3 kDa in size. Once the samples were concentrated, a buffer exchange was performed by adding 1 ml of 2M urea to the tube and centrifuging the sample for 2 hours. A total of 2 buffer exchanges were performed. Once the samples were concentrated, a BCA assay was performed again in an effort to quantify the extent of the protein removal.

6.2 Results of IgY-R7 Immunodepletion of Rat Samples

Testing of the IgY column used rat serum purchased commercially from Sigma-Aldrich. The serum was enriched according to Proteome Lab protocols and the 1st Generation FT1, FT2, W, and E fractions were labeled as Run 1 and a protein assay run to measure the protein concentrations. Afterwards, the 1G FT1 and FT2 fractions were combined and concentrated to give a single 1G FT fraction. This fraction was then diluted with Proteome Lab's Dilution Buffer to a final volume of 500 µL and run through the IgY column a second time. The 2nd Generation (2G) FT1, FT2, W, and E fractions from the

further enriched serum were collected, labeled as Run 2, and assayed for protein concentration (Figure 16).

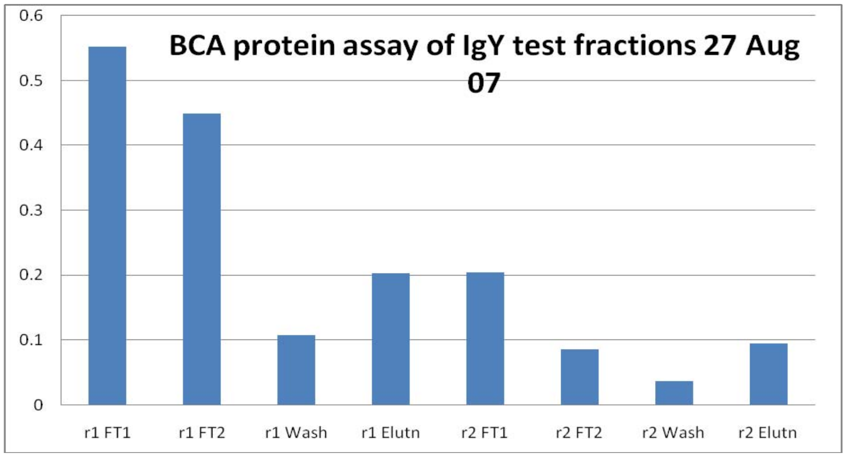


Figure 16: BCA protein assay used to measure the three fractions collected after enriching rat serum using an IgY antibody column on 27 Aug 07.
The Run 1 (R1) label designated the FT (flow through), W (wash), and E 1G (eluate) samples collected after initially enriching whole rat serum. The Run 2 (R2) label designated the 2G FT, W, and E samples collected after further enriching the R1 FT fraction.

The low concentration values from the BCA assay were of concern so to ensure that no interfering substances distorted the assay, a Non-Interfering assay was repeated on the samples to confirm the results. The NI assay results were similar enough to the BCA assay results that one can assume the values accurate (Figure 17).

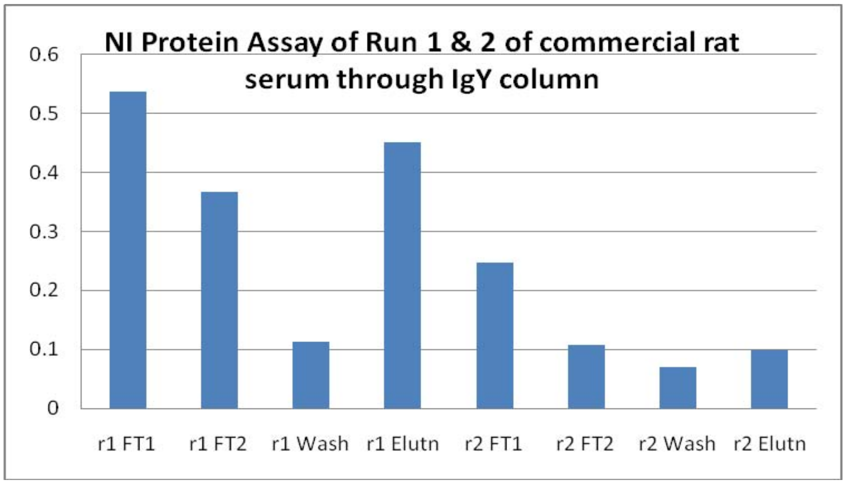


Figure 17: NI Protein Assay of commercial rat serum enriched by IgY column, repeat of BCA assay on sample runs 1 & 2.

In order for the samples to be separated by DIGE gels, the samples are required to have a concentration of 1 μg/μl at minimum however, the higher the concentration then the better. In order to figure out how much raw sample was needed for enrichment, the individual samples were concentrated once using the YM-3 spin cartridge and a NI assay was repeated.

Each fraction, FT 1, FT 2, W, and E, were individually measured after being concentrated. The values did not fit expected trends so another NI assay was repeated to ensure accurate results (Figure 18). The next assay contained non-concentrated samples and final, concentrated samples paired together. The samples combined both FT fractions into one concentrated sample and combined all four E fractions into one concentrated sample.

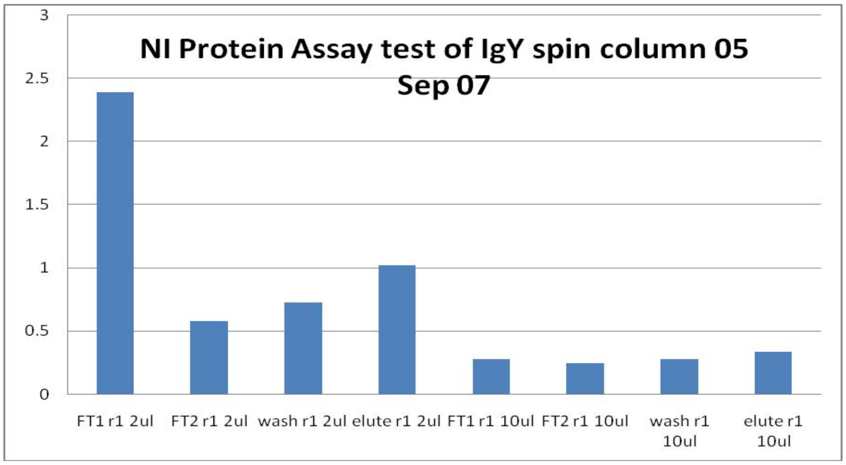


Figure 18: NI Protein Assay of commercially purchased rat serum enriched using IgY spin column and concentrated using the YM-3

The results (Figure 19) demonstrated a significant increase in protein concentration and that the IgY column was effective at removing abundant proteins as evident by the elevated values from the condensed E fractions relative to the condensed FT fractions.

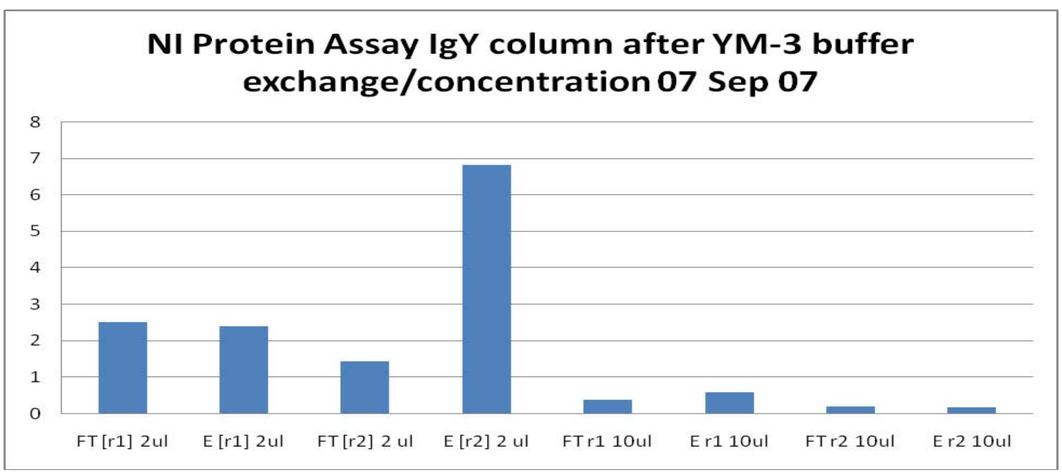
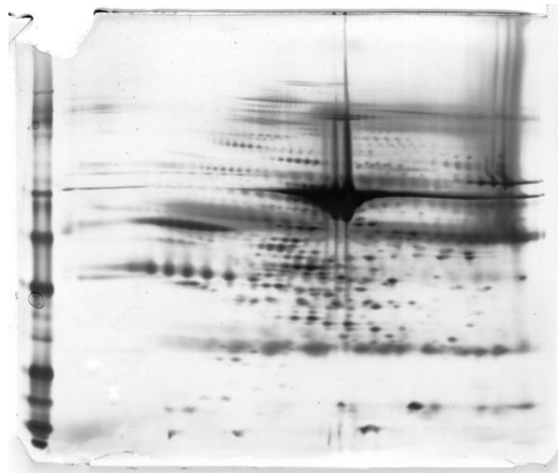


Figure 19: NI protein assay comparing IgY column enriched samples after- and before- concentration using YM-3 spin cartridge.
The first four columns indicate samples after concentration and the last four columns indicate samples before concentration.

After measuring the protein concentrations of the enriched samples, small 2D PAGE gels were run in order to provide a visual comparison of the protein resolution provided by each column. The small 2D gels were chosen as a qualitative test because they gave cheaper and faster results. To run the gels, the samples were prepared by first concentrating the FT and E fractions then exchanging the buffer using a Microcon YM-10 spin cartridge and 2M urea. The buffer exchange was deemed necessary since the IgY kit uses a buffer that is very high in salt (150 mM NaCl). This high salt content would destroy any subsequent 2D gels that may be run.

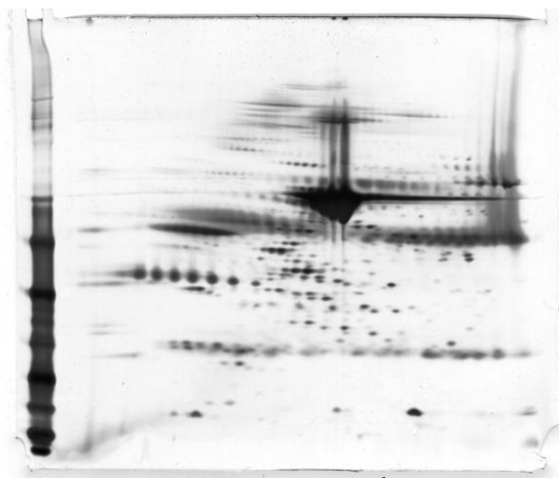
Despite the buffer exchange and protein concentration, the 2D gels showed better resolution of protein spots than the large gels of Agilent enriched samples (Figure 20). A wide range of proteins were evident based on both isoelectric point and molecular mass. The important result seen in the gels was that the Elution fraction does show increased removal of significant amounts of over abundant proteins like albumin, transferrin, and IgG. A visual comparison clearly showed a greater amount of protein in the albumin range in the E gel than the FT gel. Unfortunately, the 2 D gels still showed a lot of streaking and low resolution of protein spots. Other areas of concern were the numerous spots in the elution gels directly under the albumin blob and there appeared to be more proteins in the gel than the seven targeted over-abundant proteins listed: albumin, IgG, transferrin, fibrinogen, IgM, alpha 1-antitrypsin, and haptoglobin. The protein spots that appeared over multiple pH ranges and molecular weights could just be artifacts of degradation or other isoforms for the same proteins. The 2D gels were a good test of the column‘s effectiveness but still took considerable time to run. In order to monitor the IgY column for degradation, a faster qualitative method was needed to act as a quality control for the enriched samples. Thus, a 1D SDS-PAGE gel was run (Figure 21) to see if visual results would be similar enough to the small 2D gel to act as a quality control for a pilot study.

A: FT Fraction, Rat Serum, 1st Gen



B: E Fraction, Rat Serum, 1st Gen

C: FT Fraction, Rat Serum, 2nd Gen



D: E Fraction, Rat Serum, 2nd Gen

Figure 20: Small 2D PAGE gels comparing 1st Generation FT and E fractions and 2nd Generation FT and E fractions after buffer exchange and YM-3 concentration to demonstrate the increased enrichment with each pass through the IgY spin column.
A) Flow Through Fraction, rat serum, 1st Gen B) Elution Fraction, rat serum, 1st Gen
C) Flow Through Fraction, rat serum, 2nd Gen D) Elution Fraction of rat serum, 2nd Gen

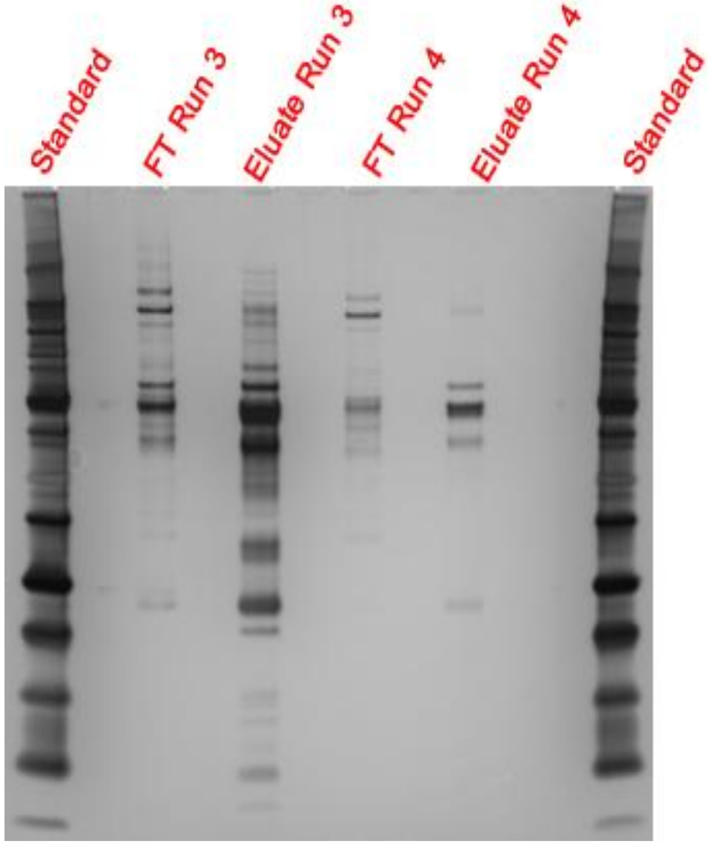


Figure 21: 1D gel of Run 3 and 4 FT and E fractions after enrichment with IgY spin column

The protein assay showed that there was 9.4 times the amount of protein in the Run 3 E fraction than in the Run 3 FT fraction. The greater amount of protein in the E fraction meant that most of the abundant proteins were removed in the first pass through the IgY column. The assay showed that the serial dilution worked well and removed additional abundant proteins as evidenced by more protein in the Run 4 FT fraction than in the Run 4 E fraction.

Once the protein concentrations were known, the fractions were run on a 1D gel and visualized using the genomics silver stain kit. Given the concentrations, 1 ul of each sample was used on the gel to see relative quantities. Figure 22 shows the four fractions in the following order: Run 3 FT, Run 3 E, Run 4 FT, Run 4 E.

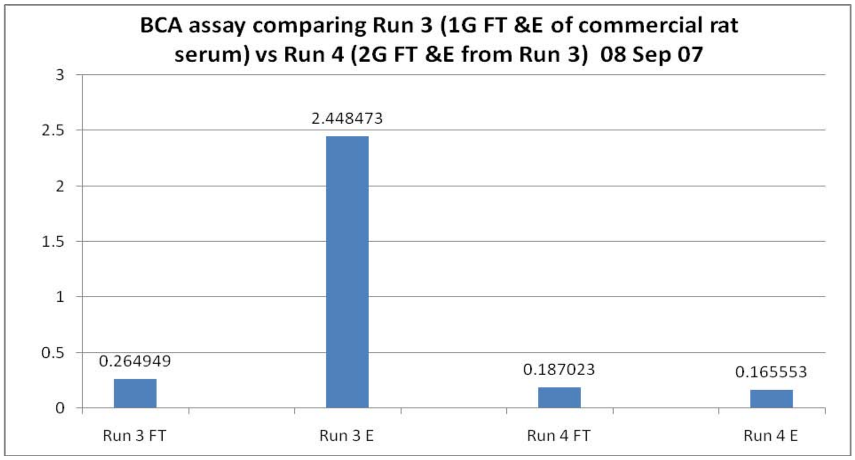


Figure 22: Average protein values for Run 3 and 4 after concentration using BCA assay (in ug/ml)

The results showed that a 1D gel was an acceptable method of testing enriched samples since there was a clear difference in proteins based on the darkness of the stained protein bands. By combining the 1D gel data with protein assay data, the IgY columns could be suitably monitored for effectiveness throughout the pilot and full studies.

The last step in characterizing the effectiveness of the IgY column was to directly compare the IgY enriched samples versus the Agilent enriched samples with one NI assay (Figure 23).

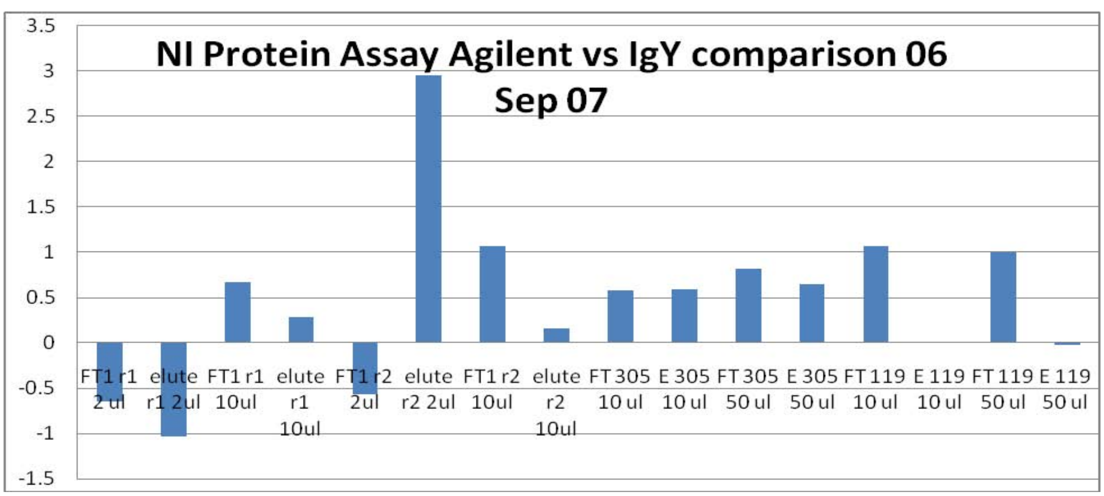


Figure 23: NI Protein assay comparing IgY enriched samples versus Agilent MARS enriched samples.

Unfortunately, this assay did not provide relevant data as many of the calculated concentrations were found to be negative in value. The results from this assay were discarded since the other assays support the use of the IgY column as a suitable separation method for the study.

6.3. Discussion of IgY-R7 Immunodepletion

Based on the results of the protein assays, 1D gels, and 2D gels, it was evident that the IgY spin column would successfully enrich the rat serum samples for use in this project. Based on the values from the protein assays, the samples required two passes through the column in order to remove enough of the over abundant proteins. To make up for the low protein concentrations, several aliquots would need to be enriched and concentrated using the YM-3 spin cartridges. To ensure all steps of the study would work as planned, a pilot study was initiated. All steps in the study were tested for optimal results using four samples, a control and high dose from Puromycin and D-Serine Study 1. Originally the pilot study called for a control and high dose from BEA as well but the protein concentration was not high enough to use the samples after enrichment and concentration.

7. SERUM PROTEIN BIOMARKER DISCOVERY: PILOT STUDY

The pilot study was an opportunity to define the optimal methods needed to ensure the IgY column removed the most amount of overabundant protein possible while still having enough sample to perform multiple DIGE gels. The Pilot Study samples were chosen from each drug study at a high dose and a control using the Terminal Sacrifice time points.

7.1 Pilot Study Methods

From each sample, 15 µl of serum was pipette into an eppendorf tube and diluted with 485 µl of IgY dilution buffer. The IgY dilution buffer was made by diluting 100 µl of Sigma protease inhibitor cocktail to 10 ml with IgY dilution buffer. The diluted serum sample was then run through the IgY column which produced the 1st Generation (1G) of FT, Wash, and E fractions (~1.5 ml total). The 1G FT and E fractions were concentrated using the Microcon YM-3 spin filters in the Sorvall centrifuge (3200 x g at 35 min, 4 C). Once the fractions were concentrated, the 1G FT samples were removed and mixed together by drug dose to create a pooled sample. The pooled sample was then diluted with IgY dilution buffer plus protease inhibitor to give a final volume of 500 ul. This pooled, 1G FT fraction was run through the IgY column once more to create the 2nd Generation (2G) of FT, Wash, and E fractions. These 2G FT and E fractions were concentrated using the Microcon YM-3 spin filters as before and protein concentrations determined using the BCA assay (Figure 24). Dosed and control samples were taken from three nephrotoxin studies (Table 3).

Table 3: Animal subjects by dose and drug for pilot study using IgY column

	D-Serine		Puromycin		BEA	
Dose	Control (0mg/kg)	500 mg/kg	Control (0 mg/kg)	500 mg/kg	Control (0 mg/kg)	500 mg/kg

Rat subject number	243	247	303	320	272	285
	246	251	305	324	274	289

7.1.2 CyDye Labeling of Proteins

All work with the Cy Dyes must be done in as minimal light as possible since the dyes are light sensitive. All work from labeling to 1st and 2nd dimension separation of the proteins must be done with the lights off or with the experiments covered. First, the Cy Dyes needed to be reconstituted so once they were removed from the freezer and allowed to slowly warm for 5 minutes, 5 µL of anhydrous DMF was added to the lyphohilized powder to create a 1 mM stock solution. The solution was vortexed for 30 seconds then centrifuged for 30 seconds at 12000 x g. Working solutions were made from the stock solution by adding 1.5 volumes (7.5 µL) of DMF to a new eppendorf tube then mixed with the dyes. This dilution creates a final working solution of 400 pM which is recommended for labeling 50 µg of protein. Next, the pH was raised to the optimal value of 8.5 by adding diluted 50mM sodium hydroide and pH paper to check. After prepping the Cy Dyes, the samples were labeled by adding 1 µL of Cy Dye to the eppendorf tube using Cy 2 to label the internal standard, Cy 3 to label the control sample, and Cy 5 to label the experimental sample. Once the samples were labeled the tubes were vortexed and centrifuged for 30 sec each and incubated on ice for 30 minutes. After labeling, 7 µL of Pharmalyte buffer was added to each Cy Dye tube to quench the labeling reaction. Next, Rehydration Buffer (RHB) was prepared by adding 30 mM DTT and a calculated amount was added to the Cy Dye labeled samples to give a final volume of 345 µL. The samples, now labeled and in RHB, were kept in their eppendorf tubes and floated in a water sonication bath for 1 minute then placed on a shaker table for 15 minutes. The cycle of 1 min sonication, 15 minute shaker table, was repeated for a total of four cycles. The exact volumes of Cy Dye added to each sample are listed in Table 4.

Table 4: Example of volume of enriched, pooled sample added to Cy Dye for labeling

50ug each	Cy2	Cy2	Total Cy2	Cy3	Cy5
Comparison 1					
305/303 control vs. 320/324					
303/305 volume added	67.7			135.4	
320/324 volume added		33.2			66.5
Comparison 2			100.9		
243/246 control vs. 285/289					
243/246 volume added	39.6			79.3	
285/289 volume added		56.6			113.3
			96.2		

7.1.3 Protein Separation in the 1st Dimension

After the samples were labeled, Isoelectric Point Gradient (IPG) strips were prepared to separate the serum proteins by isoelectric point in a pH gradient ranging from 4 to 7. First, the labeled samples were pipetted into the lanes of a re-swelling tray. Next, the plastic liners were removed from the IPG strips and the strips placed into the re-swelling tray with the plastic backing facing down. Mineral oil was added on top of all the IPG strips to prevent them from drying out, the cover was placed on the re-swelling tray to protect the samples, the whole tray was wrapped in black plastic to block light, and lastly the IPG strips were allowed to incubate overnight to absorb all the proteins. The next day the IPG strips were carefully removed from the trays using tweezers, excess mineral oil was allowed to drip off onto lab wipes, and the strips were placed into a liner tray. Two electrode strips were cut to fit across the liner tray and 1 mL of water was pipetted onto each side of the electrode strip with excess water blotted off using lab wipes. The electrode strips were placed across the IPG strips at the top and bottom then the plastic electrodes clamped in place. The electrode/IPG tray conglomerate was clamped in place on the electrophoresis apparatus and mineral oil was used to fill any remaining gaps. The apparatus and its cooling system were turned on, set to 300V, and allowed to run overnight in an 18 hour gradient cycle reaching a max of 3000V.

As the serum is separated in the 1st dimension, the gel tank and necessary buffers were prepared for running the gel in the 2nd dimension the next day. Previously mixed stock buffer solutions in 10X concentrated form were prepared and kept in a cold room. The lower tank buffer was prepared by pouring 8 L of Millipore water into the lower tank and mixed with stock buffer, which was diluted by mixing 1 L of 10X concentrate with 1 L of Millipore water in a 2 L graduated cylinder. The diluted solution was left in the tank overnight with the cooling fan turned on and the tank covered overnight. The Upper buffer was prepared by diluting 300 mL of 10X Upper buffer solution to 3 L in a 4 L Erlenmeyer flask with Millipore water. The diluted solution was transferred to a 3 L plastic beaker and placed on a stir plate with stir bar and covered for the evening.

7.1.4 Protein Separation in 2nd Dimension

The next day, the samples and apparatus were ready for separating the serum proteins in the 2nd dimension. First, two 50 mL conical tubes of equilibration buffer were taken from the cold room and allowed to thaw. To one tube was added 800 mg of DTT and to the other tube was added 1 g of iodoacetamide (IAA). The IAA tube was wrapped in aluminum foil since IAA is light sensitive. Both conical tubes were placed on a rocker plate to warm and dissolve the newly added chemicals. Once thoroughly mixed, the equilibration buffer/DTT and equilibration buffer/IAA solutions were filtered in their own disposable 0.22 micron vacuum filter beakers (covering the IAA beaker in aluminum foil again) to remove any particulates. In the mean time, the IPG strips were removed from the electrode tray using tweezers and labwipes to blot off excess mineral oil. The strips were placed into a re-swelling tray, covered with the DTT buffer, wrapped in aluminum, and placed on a rotating shaker (as before) for 15 minutes. After the 15 minutes, the DTT buffer was vacuumed up and the process repeated three more times, once more with DTT and twice with the IAA solution giving a total of four washes in equilibration buffers. Next, the gel apparatus was prepared by placing spacers into the slots in the upper tank and double paned, pre-cast gels purchased from Nextgen Sciences Inc (cat # PRS-703696) were placed into the tanks as well. Then, the Upper buffer was poured into the upper tank until the tops of the gels were barely covered, the IPG strips placed into the gel wells using tweezers, and the remaining buffer poured into the tank.

The apparatus was enclosed and the gel run at 500 mA for 5 hours, 51 minutes. Once the proteins had separated, the gels were removed from the apparatus, cut from the glass plates, placed in Fix 1 solution (50% EtOH, 10% Acetic Acid, 1L total) for 30 min, and then left in Fix 2 (30% EtOH, 1 L total) overnight.

7.1.5 Fluorescent Gel Scanning and Silver Staining

The next day the gels were removed from Fix 2 and washed with Millipore water for 5 min in preparation for scanning. Each gel was placed on the glass plate from the scanner, a FUJI FLA-5100, and the gel fluorescently scanned: Cy 2 scanned at 437 nm with bpb1 filter with aperture, Cy 3 is scanned at 532 nm and modified Cy3/Cy5 filter from FUJI with aperture, and Cy 5 scanned at 635 nm with LPR filter with aperture. All scans were saved as 16 bit images at 100 μm resolution with photomultiplier voltages at 400V.

After fluorescent scanning, the gels were silver-stained using a kit from Genomic Solutions. First, the gels were placed into Fix 1 which consisted of 1.6 L of ethanol and 400 ml of acetic acid mixed and diluted to 4 L with Millipore water for 1 hour. Next, the gels were placed into Fix 2 which consisted of the dry chemicals in the Proprietary Fix 2 bottle and 1200 ml of ethanol mixed and diluted to 4 L with Millipore water for 1 hour. The glutaraldehyde included in the kit was omitted from the solution since the glutaraldehyde would interfere with mass spectroscopy identification. Then, the gels were washed a total of four times in Millipore water, each wash was 15 minutes in length. While washing in water, the Silver Nitrate Solution was prepared by mixing the liquid in the Silver bottle with 1 ml of formaldehyde and diluting to 4 L with Millipore water. After washing in water, the gels were placed in the Silver Nitrate Solution for 45 minutes followed by a 1 minute wash in Millipore water. After washing, the gels were placed in Developer solution for 5 to 8 minutes. Developer solution was prepared by mixing the bottle of dry chemical with 0.6 ml of formaldehyde and diluting to 4 L with Millipore water. Lastly, Stop Solution was mixed using the bottle of dry chemicals and 80 ml of acetic acid all diluted to 4 L with Millipore water. The gels were left in the final Stop

solution for 10 minutes. After silver staining the gels, each was digitally scanned and the images saved to the network drives for later analysis.

Once the gels were fluorescently scanned, the images were imported into the ProGenesis PG240 SameSpots software package. This software allows the user to match the Cy 2 labeled internal standards from each gel relative to a single reference gel. The software then measures the difference in fluorescent intensity between the Cy 3 labeled control spots and the Cy 5 labeled disease state spots. After the protein spots were aligned, the SameSpots software identified approximately 3,700 individual spots. However, many of the spots were artifacts or background staining so each spot was individually reviewed for intensity, shape, location, and area to mask false spots so the software would only compare true proteins. The lists of true spots are shown in Figures 33 and 34. The software used various statistical devices such as Principal Component Analysis (PCA) to determine which spots had the greatest difference in fluorescent intensity which was directly related to volume of protein in a spot.

7.1.6 Mass Spectroscopy Identification: In Gel Digestion Protocol

The first step in obtaining protein identifications was to excise the spots from the gel. To do this each gel was placed on a light box and a print out of the silver stain images with the protein spots of interest labeled was tacked to corkboard on the wall. By carefully comparing the labeled print outs with the gels each spot was cut out using a razor blade, placed in an eppendorf tube, then stored in the refrigerator. After removal of all protein spots of interest, the gel slices (containing the proteins) were digested into peptide fragments using the enzyme trypsin.

To initiate the tryptic digest the necessary stock solutions were prepared, and the final volumes depended on how many samples were to be digested. A 30 mM Potassium Ferricyanide (PFC) solution was made by combining PFC powder from Sigma with Millipore water in a 49.4 mg/5ml ratio in a 15 ml conical tube. 100 mM Sodium Thiosulfate (STS) combined powdered STS from Sigma with Millipore water in a 79.1mg/5 ml ratio in a 15 ml conical tube. 1M Ammonium Bicarbonate (ABC)

combined powdered ABC from Sigma with Millipore water in a 0.79 gm/10 ml ratio in a 15 ml conical tube. From the stock 1M ABC solution came the 75 mM, 50 mM, and 25 mM ABC working solutions. The working solutions used 750 µl, 500 µl, and 250µL in separate conical tubes and diluted to 10 ml using Millipore water. The final two solutions, DTT and iodoacetamide (IAA), were made right before use with the IAA kept wrapped in aluminum foil. 10 mM DTT was made by combining powdered DTT from Sigma with 25 mM ABC in a ratio of 1.5 mg/1 ml in a 1.5 ml eppendorf tube. 55 mM iodoacetamide was made by mixing powdered IAA from Sigma with 25 mM ABC in a ratio of 10 mg/1 ml in a 1.5 ml eppendorf tube.

Day One of the ‗In Gel' Digestion protocol began with destaining the gel pieces. First, Farmers reagent was made by combining 30 mM potassium ferricyanide and 100 mM sodium thiosulfate in a 1:1 ratio in a 15 ml conical tube. Next, 50 µL of Farmers Reagent was added to each gel piece in its eppendorf tube and allowed to incubate approximately 3 – 5 minutes until the silver stain was removed but before the gel pieces became a darker yellow than the Farmers reagent. After incubating, the Farmers supernatant was removed and 60 µL of Millipore water was added and incubated for 10 minutes after which the supernatant was removed.

Next, the gel pieces were washed and dehydrated to shrink the polyacrylamide gel material so during the next stage, reduction/alkylation, the DTT and IAA would be evenly exposed to all of the proteins. The Washing Step started by adding 50 µL of 25 mM ABC for 10 minutes in a vortex block then removing the supernatant. Then, 50 µL of a working solution of 50 mM ABC and 100% Acetonitrile (ACN) mixed 1:1 was added, incubated for 10 minutes in a vortex block, and then the supernatant removed. The previous step with 50 µL of 50mM ABC:ACN was repeated once more. Extra washes were occasionally necessary to get the gels from large, translucent pieces to small, opaque white pieces which indicated all excess water had been removed. To ensure all water had been removed, the gel pieces were dried in a speed vacuum for approximately 10 minutes.

Once the gel pieces were dehydrated, the proteins inside were reduced and alkylated to unfold the proteins so the trypsin could evenly digest all of the protein. Reduction of the disulfide bonds in the proteins was done by adding 20 μL of 10 mM DTT in 25 mM ABC and incubated for 60 minutes at 37^{o}C in a heating block containing water. Afterwards, the sample was alklyated by adding 20 μL of 55 mM IAA in 25 mM ABC directly into the eppendorf tube without removing the DTT solution. The IAA was left to incubate for 45 minutes 37^{o}C in the dark in a heating block containing water as well.

Another washing/dehydration of the gel pieces was conducted to allow the trypsin to be able to digest as much of the protein at once in an even manner. First, 50 μL of 25 mM ABC was added to the tubes, incubated for 10 minutes in a vortex block, and then the supernatant was removed. Next, 50 μL of the 50 mM ABC:ACN (1:1) solution was added, incubated for 10 minutes in a vortex block, and then supernatant was removed. The previous step was repeated once more, occasionally twice more until all the gel pieces were small and opaque white in color. Lastly, the gel pieces were dried in a speed vacuum for approximately 10 minutes.

Once the gel was dried and shrunk, the proteins in the gel were digested with the trypsin enzyme. First, one vial of lyophilized trypsin was dissolved with 200 μL of 50 mM acetic acid and mixed well. The stock solution was divided into 12.5 μL aliquots and stored in 0.5 ml eppendorf tubes and stored in the -20^{o}C freezer. One of the 12.5 μL aliquots was thawed and diluted with 112.5 μL of 75 mM ABC. Then, 5 μL of the diluted trypsin (10 ng/μL in 75 mM ABC, pH ~7.6) was added to the gel pieces and incubated at 37^{o}C for 20 min in a water bath. After incubating, 45 μL of 25 mM ABC was added to the tube and then incubated at 37^{o}C overnight in a water bath.

Day 2 of the process was the recovery of the peptide fragments, using μZip Tips to place the peptides on a MALDI plate, followed by MALDI-TOF/TOF sequencing, and finally MASCOT database identification. Recovery of the peptides began with prewashing 1.5 ml eppendorf tubes with 200 μL of 0.1% tetrafluoroacetate (TFA) in 60%

acetonitrile (ACN) then removing the supernatant. Next, the digested peptides from Day 1 were removed from the water bath and centrifuged. The supernatant from the gels was removed and placed into the newly washed eppendorf tubes. To the dried gel pieces was added 30 µL of 0.1% TFA in 60% ACN and then the supernatant removed and placed into the new eppendorf tube. The previous step was repeated 3 more times but with 10 minute incubation in a vortex block before recovering the supernatant and placing it in the new eppendorf tube. The final step dried the extracted peptides in the new tubes to a final volume of 10 – 15 µL using a speed vacuum but being careful ***not*** to dry to completion. If the samples did dry out completely, the peptides could still be recovered if 0.1% TFA in 60% ACN was added and the samples speed vacuumed down to 10 – 15 µL again.

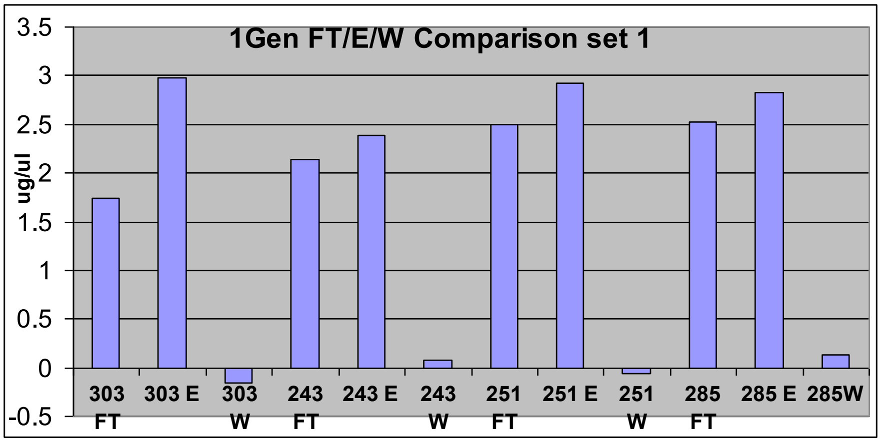
Once the peptides were concentrated, the samples were prepared for plating on the MALDI plate using the µZip Tips. The µZip Tips are 10 µL pipette tips that use resin beads to provide a scaffolding substrate for protein peptides to bind as the digested mixture passes over the beads. The resin beads are conglomerates of organic chains 18 carbon atoms in length which provide consistent pore sizes to capture the peptides. The proteins are bound to the tips then eluted into a matrix that contains the peptides until analysis by MALDI mass spectrometry. The process began by setting up two glass vials, one vial with 100% acetonitrile (ACN) and the other with 60% CAN, 0.3% TFA, as well as a 1.5 ml eppendorf tube with 0.3% TFA in Millipore water. Additionally, a new eppendorf tube was set up for each sample which contained 2.5 µL of 60% ACN 0.3% TFA. A fixed 10 µl pipette was used since the tips must remain wet once the process is initiated as air will dry the beads and prevent peptide capture or, if peptides are already captured, prevent their elution into the matrix. The protocol began with pipetting 100% ACN to prepare the Zip Tip, disposing of the ACN into a waste jar, and repeating with 60% CAN, 0.3% TFA solution. Next, the Zip Tip was equilibrated with two washes of 10 µL 0.3% TFA. Then, the concentrated peptides extracted earlier were bound to the column by pipetting the sample up and down 10 times through the equilibrated Zip Tip. An aliquot was placed at the top of the tube and the rest of the sample pipetted through the column to ensure all the peptides came into contact with the Zip Tip. The captured peptides were washed twice with 10 µL 0.3% TFA to remove salts and contaminants.

Next, the peptides were eluted by pipetting the aliquot of 60% ACN 0.3% TFA into the new tube. The eluted samples were placed into the SpeedVac until the pressure dropped to 750 mTorr as read by the digital gauge. This left a remaining volume of approximately 0.5 μL in the tube. After this procedure, the Matrix Solution was made, and it is important that this solution be made fresh 2 - 3 days prior to use to ensure accuracy on the MALDI. The Matrix Solution was made by dissolving α-cyano-4-hydroxy-cinnamic acid in 60% ACN/0.1% TFA plus 10 mM Ammonium Phosphate monobasic to a final concentration of 5 mg/ml. Next, 1 μL of Matrix Solution was mixed with the 0.5 μL in the tube by pipetting up and down then spotting on the MALDI plate. The last step was to make up a MALDI standard by combining 1 μL of pre-mixed standard from the freezer with 99 μL of Matrix Solution. The mixed standard was spotted on the plate before and after the samples so the MALDI could be monitored to ensure the settings remained optimized throughout the run. Once the peptides were spotted onto the MALDI plate, the Applied Biosciences 4800 MALDI-TOF/TOF was optimized and then run. This second generation of the MALDI-TOF/TOF 4800 model allows the user to control the speed of the ion before entering the collision cell where fragmentation occurs. By tuning the speed of impact, the user can determine how much fragmentation occurs allowing analysis of a wide range of daughter ions compared to the parent ion.

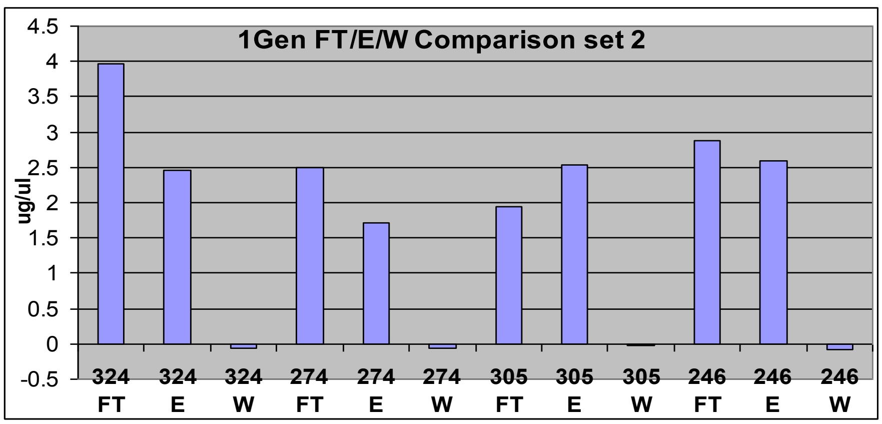
7.2 Results

After the BCA assays (Figure 24: BCA assays quantifying and comparing 1G FT/E/W fractions for all samples used in the pilot study., A-D) determined the protein concentrations in the FT and E solutions, 1D gels were run to see which proteins were evident in the samples and to see if the overabundant proteins were being removed from the FT fractions as expected. The gels were performed according to protocols provided with the Invitrogen kit. Images A and B compared the FT and E fractions of each sample, 1 ul of sample was used based on the high concentrations. The gels showed little difference between the samples, most likely because samples were not properly pipetted into each well causing the samples to flow across neighboring lanes. There were some distinctions in band intensity between some FT and E fractions, but overall every

A



B



C

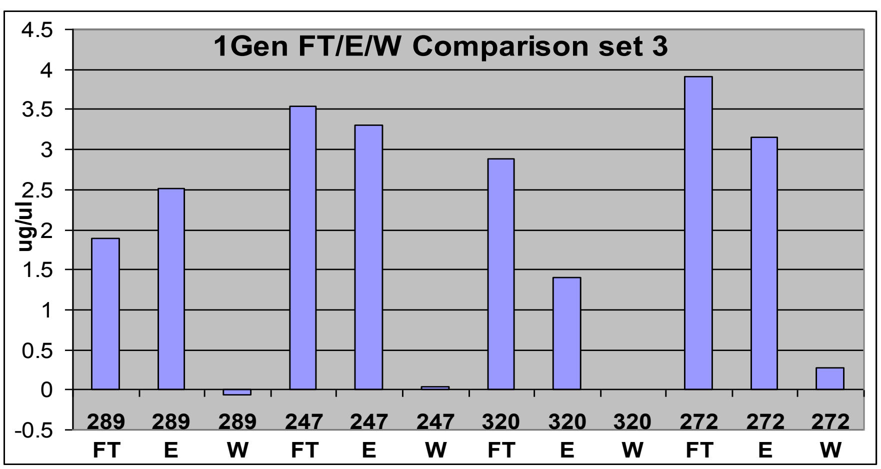


Figure 24: BCA assays quantifying and comparing 1G FT/E/W fractions for all samples used in the pilot study.

The assays should show greater values for the E fraction indicating removal of overabundant proteins. The values are used later for 2D DIGE gels.

A) Set 1 B) Set 2 C) Set 3

sample appeared the same. Because of the likely contamination, Images C and D were run with fewer samples and every other lane was intentionally skipped. Unfortunately, some lane contamination in Figure 25C and 25D was still seen. However, there were enough overall differences between the FT and E samples to conclude that overabundant proteins are being removed sufficiently enough to proceed with the Pilot Study.

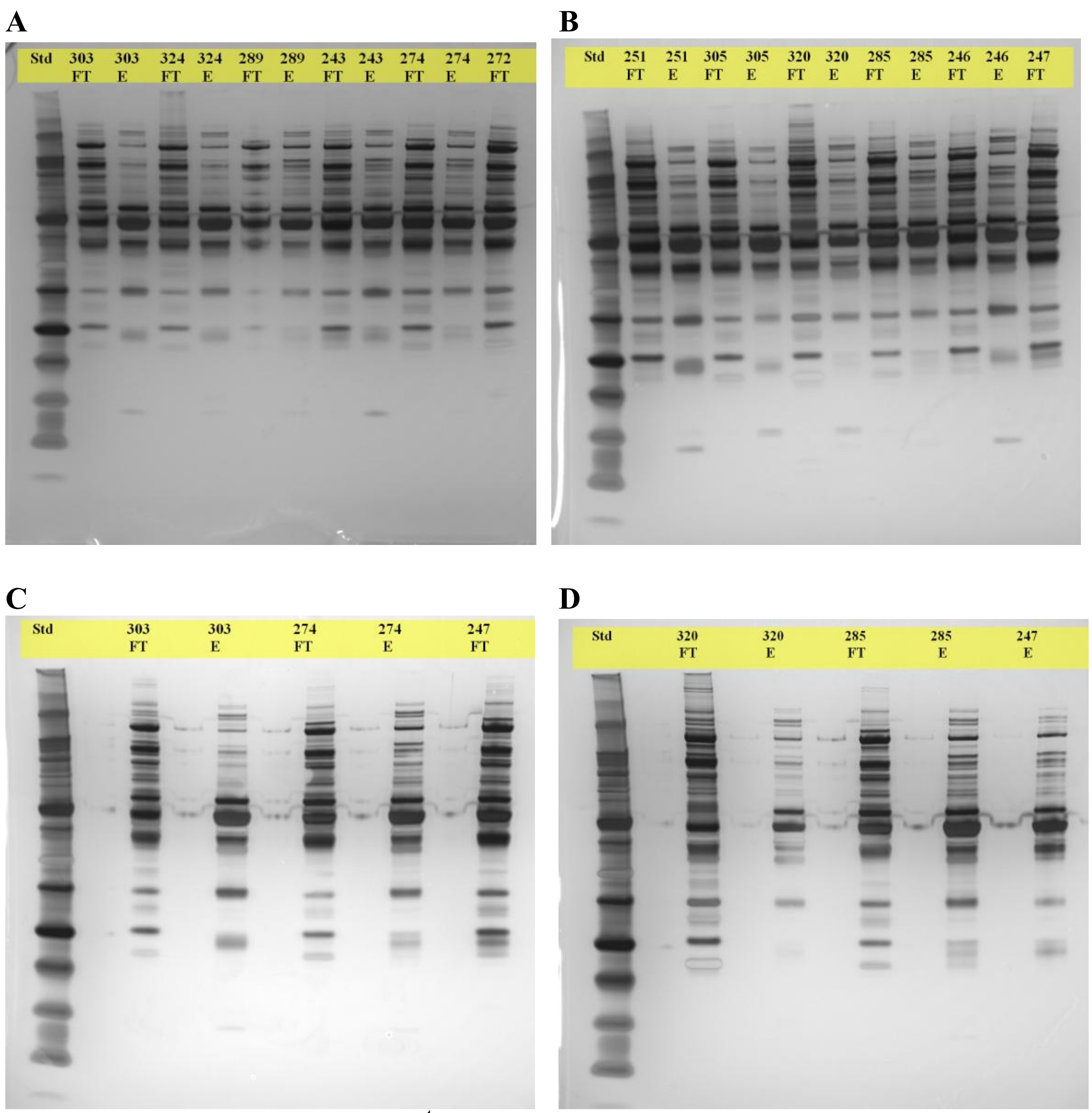


Figure 25: 1D SDS-PAGE of 1st Generation (1G) IgY enriched samples comparing FT and E fractions of pilot study samples.

A&B) All pilot study samples, gels inconclusive due to evident lane contamination. C&D) Randomly selected pilot study samples showing some lane contamination but overall enough difference between FT & E fractions to proceed with pilot study.

Once the samples were all concentrated and characterized, the condensed FT fractions were again diluted to ~500 ul with IgY dilution buffer plus protease inhibitor and run through the IgY column to give the 2^{nd} Generation Flow Through and Elution fractions (2G FT and 2G E). The samples were immunodepleted, collected, and concentrated using the same steps and equipment listed previously.

Another BCA protein assay (Figure 26) was used to quantify the final concentrations of the condensed FT and E fractions and to compare the values for evaluation of IgY column performance. The BCA assay was performed the same as previous but 15 µl of sample were used to give a 5µl/well concentration.

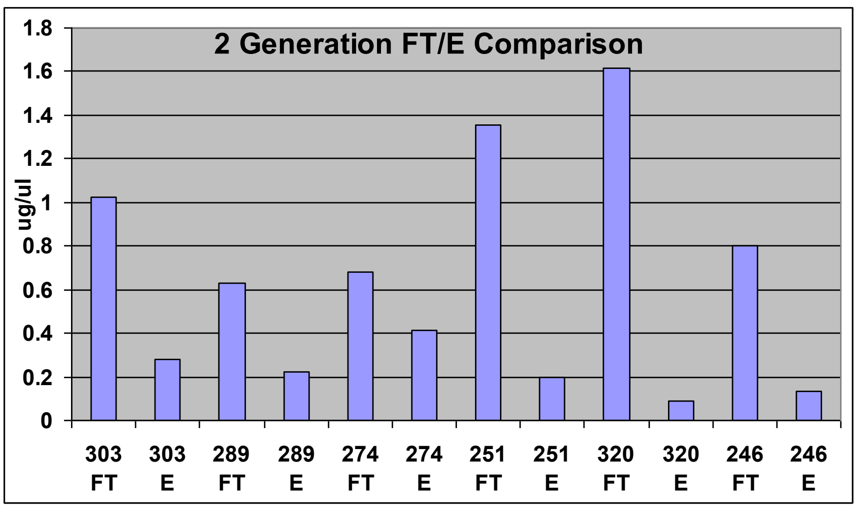
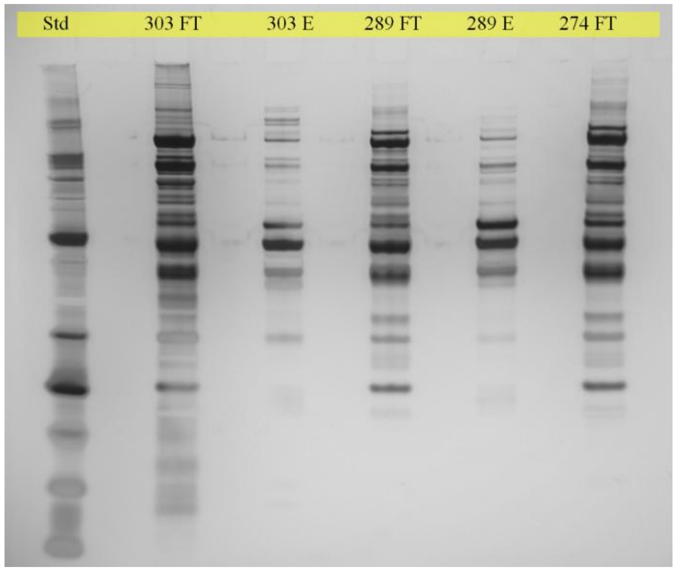


Figure 26: BCA assay quantifying and comparing 2G FT/E fractions

Based on the BCA assay values, the IgY column worked as expected and improved depletion with each subsequent FT generation. Once the BCA assay was completed, 1D gels were performed on select samples to gauge the efficiency of the IgY column. The gels were performed as previously outlined with the only difference being the use of 5 µl of sample to compensate for the lower protein concentrations (Figure 27). PAGE analysis demonstrated that most of the overabundant proteins were removed from the FT fractions and the 2D DIGE gels would work appropriately using this methodology.

A



B

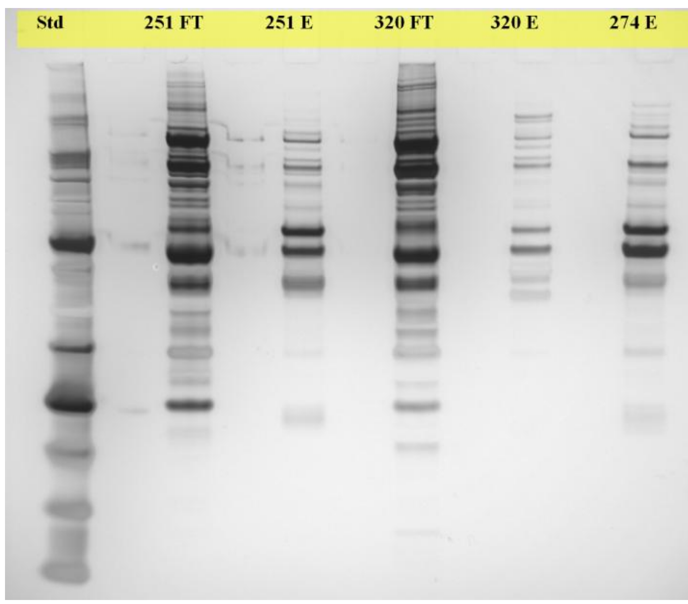


Figure 27: 1D SDS-PAGE of 2nd Generation (2G) IgY enriched samples comparing FT and E fractions of pilot study samples.

Images A&B demonstrated clear distinction between bands of over abundant proteins in FT and E fractions proving the IgY column further enriched samples with additional passes through the column.

After immunodepletion and characterization of the the pilot study samples, it was determined that the protein concentrations for the D-Serine 500 mg/kg and BEA control samples were too low for use in this study (Figure 28). In order to continue the Pilot

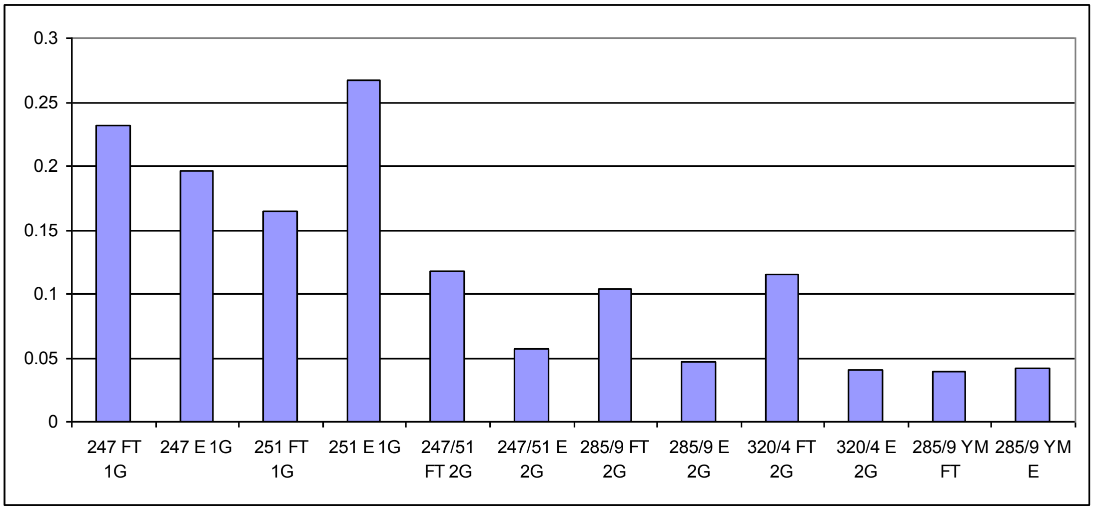


Figure 28: BCA protein assay comparing FT and E fractions from 1G and 2G of high dose samples (500 mg/kg) of d-serine, puromycin, and BEA after IgY enrichment, prior to YM-3 concentration.

Study, the D-Serine control and BEA disease state samples were mixed. The mixing of samples was deemed acceptable for this initial study since all control subjects were fed the same mixture of corn oil. The pooled pilot study samples were also tested via NI assay (Figure 29). The gels were silver stained and scanned to create images for analysis

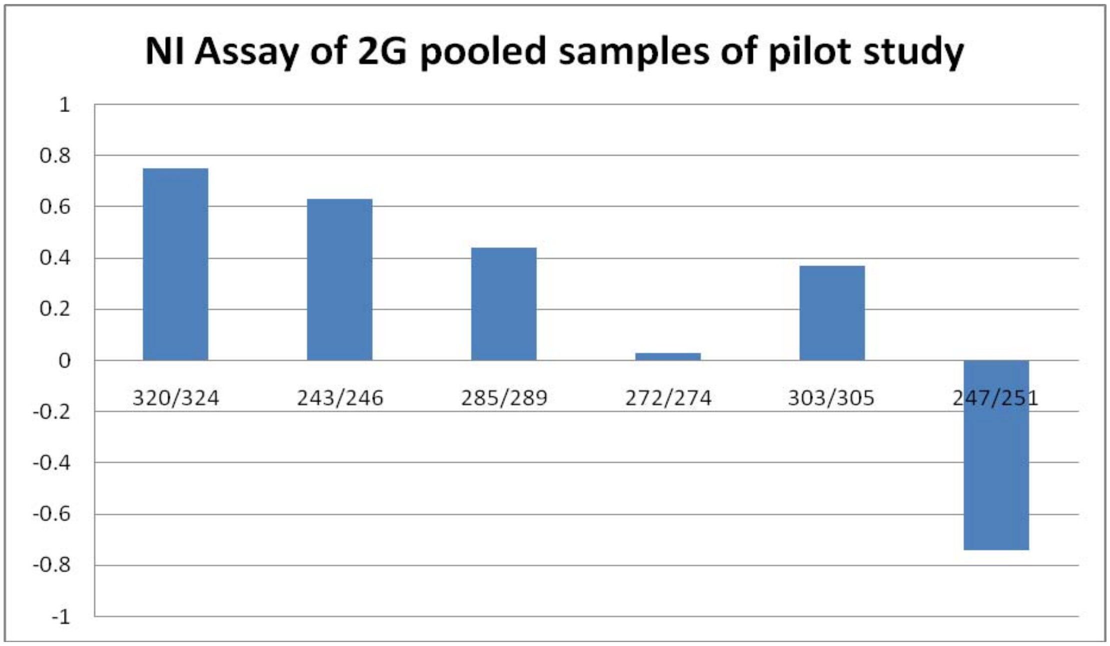
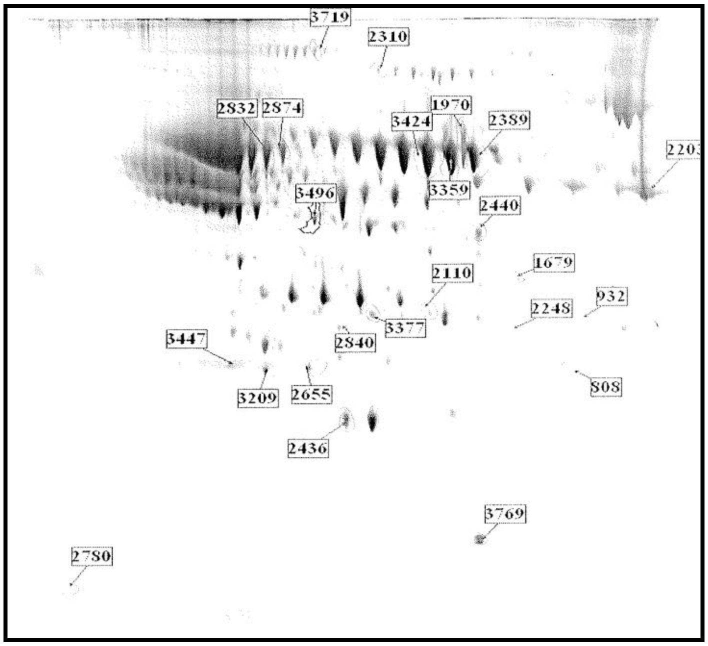


Figure 29: NI assay of 2G pooled samples of pilot study

(Figure 30). After comparing the gel images for differences in protein volume, the top protein spots that demonstrated an increase from control to disease state (up-regulated proteins) and the top protein spots that showed a decrease from control to disease state (down-regulated proteins) were identified for further analysis via peptide sequencing utilizing MALDI-TOF/TOF mass spectrometry as well as identification using the MASCOT database (Figures 30-33). The D-serine/BEA gel (243/246 versus 285/289) contained 24 spots of interest: 18 up-regulated and 6 down-regulated. The puromycin samples (303/305 versus 320/324) contained 18 spots of interest: 13 up-regulated and 5 down-regulated.

A



B

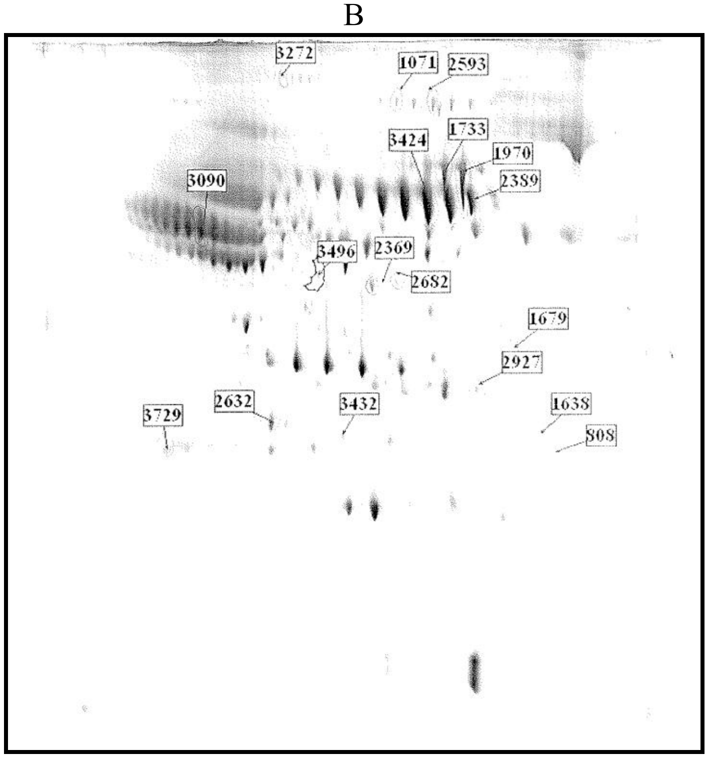


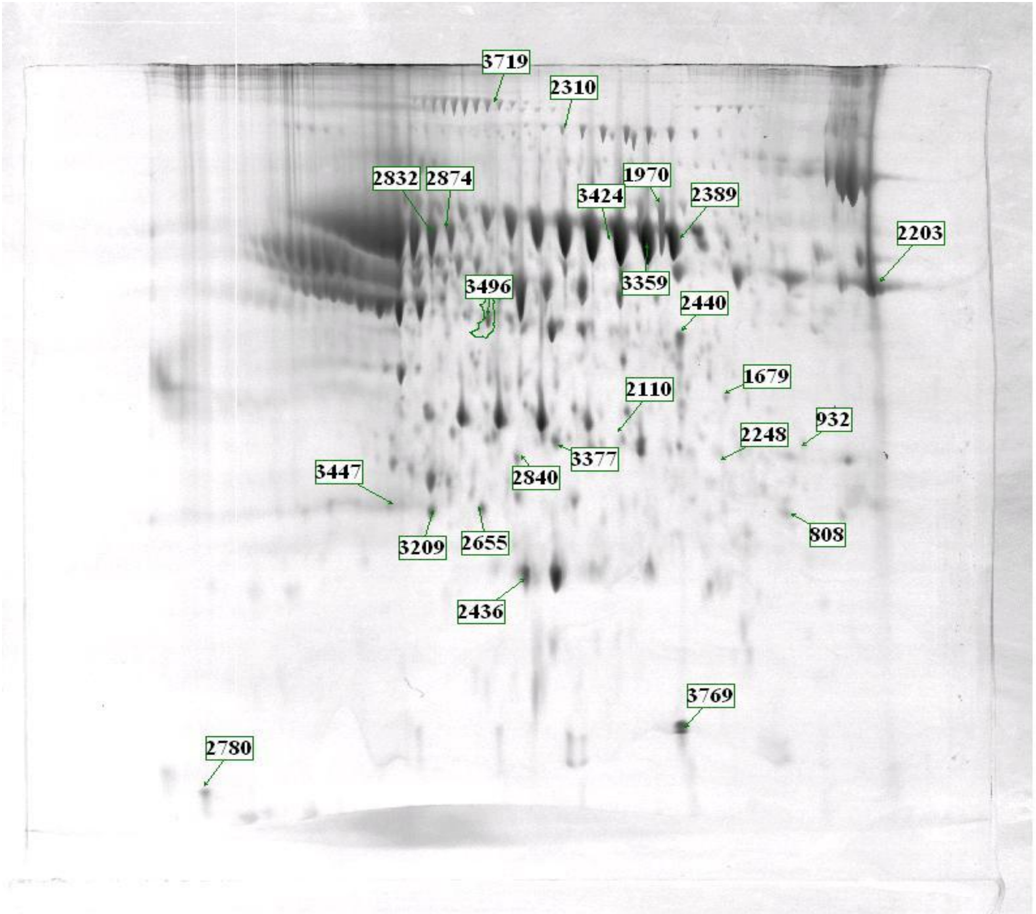
Figure 30: Pilot study 2D DIGE gel images showing up- and down-regulated spots identified as potential biomarkers using SameSpots software.

A) Gel 1 – samples 243/246 versus 285/289; 24 spots: 18 up-regulated and 6 down-regulated.

B) Gel 2 303/305 versus 320/324; 18 spots: 13 up-regulated and 5 down-regulated

Once the peptides were sequenced based on mass/charge ratio and intensity, the data was input into the MASCOT database where the software matched the identified peptides with known sequences and identified the closest match. The general criteria for a confirmed identification are a minimum overall ion score of 100 and minimum individual peptide ion score of 50. Some peptide sequences matched more than one protein so all possible proteins were listed, but a final list of 48 protein identifications were obtained (Figure 31-34).

Woolard SS (DIGE) 2D gel—243/6 vs 285/9 14 spots pilot study



Summary of Protein Identification

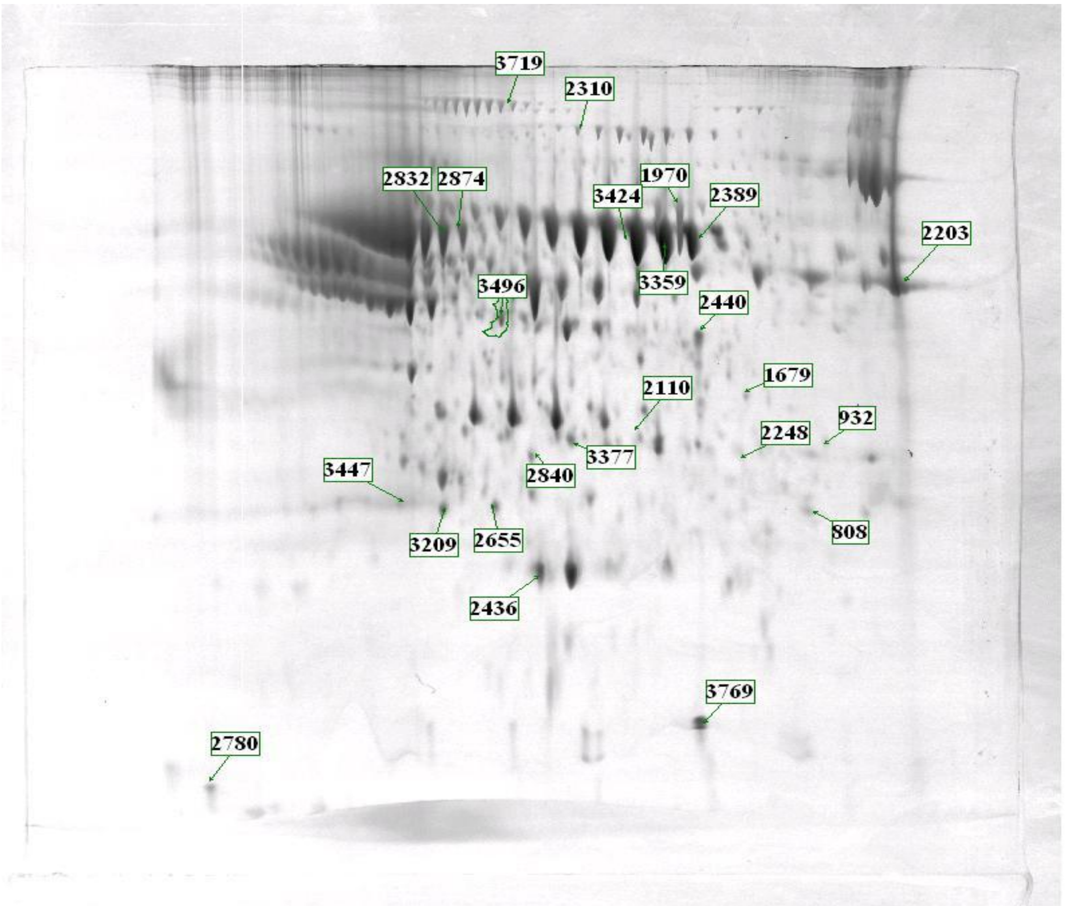
Protein identification by in gel trypsin digestion, extraction c18ZipTip and MALDI-TOF/TOF

C8- Standard — BSA
C9- Blank – Background only
C10- Spot 3719 – Inter-alpha-inhibitor H4 heavy chain--gi|126722991
C11- Spot 2310 – Da1-24 and complement factor B--gi|33086684
C12- Spot 2832 – LMW T-kininogen I precursor--gi|205085 and T-kininogen--gi|207341
C13- Spot 2874 – LMW T-kininogen I precursor--gi|205085 and T-kininogen--gi|60392582
C14- Spot 3474 – Hemopexin precursor--gi|122065203
C15- Spot 1970 – Albumin--gi|158138568
C16- Spot 2389 – Hemopexin--gi|16758014
C17- Spot 2283 – Fibrinogen B beta chain--gi|455105
C18- Spot 3496 – Fibrinogen, gamma polypeptide--gi|61098186
C19- Spot 3359 – Hemopexin precursor--gi|122065203
C20- Spot 2440 – Chain A, Rat Transthyretin Complex With Thyroxine (T4)--gi|20663827
C21- Spot 2110 – Chain A, Crystal Structure Of Rat Alpha 1-Macroglobulin Receptor Binding Domain--gi|12084772 and pregnancy-zone protein--gi|21955142 and Alpha-1-macroglobulin precursor--gi|81872093

Date of completion: 031108 Spot set 020508_09161 Runs 26-27

Figure 31: Summary of up-regulated proteins identified in IgY pilot study of d-serine control (243/246) versus BEA 500 mg/kg (285/289).
Spots 1 – 12 listed.

Woolard SS (DIGE) 2D gel—243/6 vs 285/9 13 spots pilot study



Summary of Protein Identification

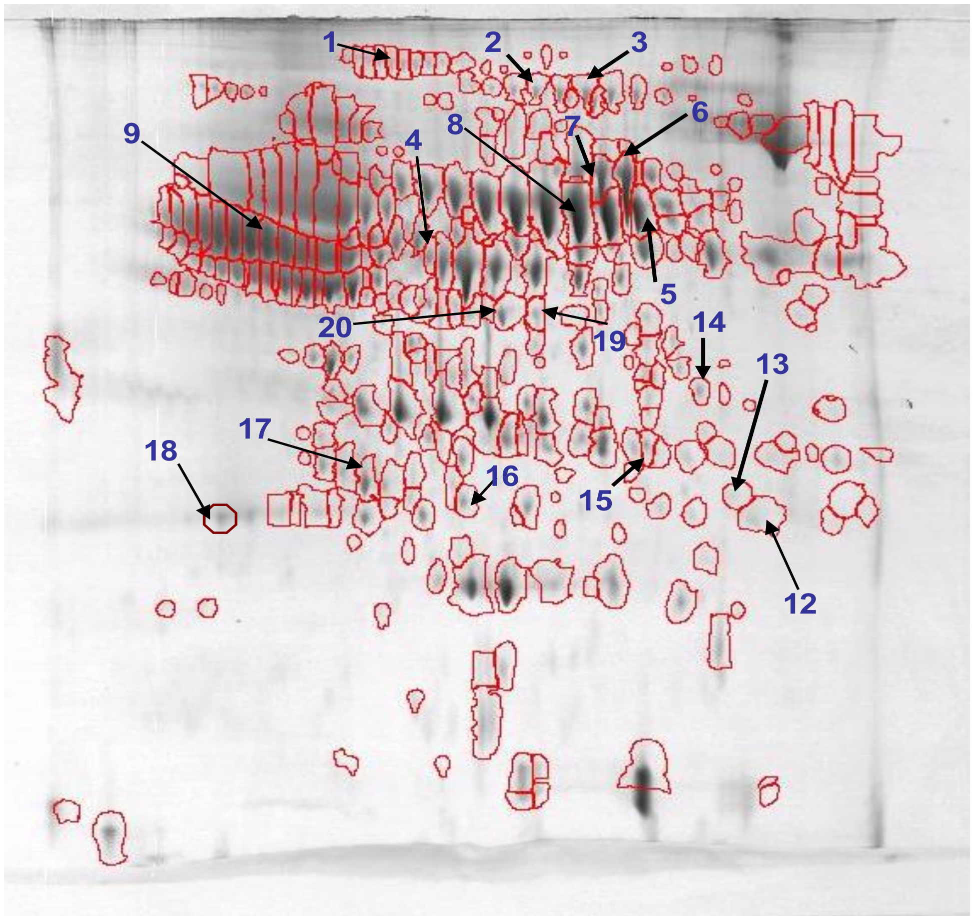
Protein identification by in gel trypsin digestion, extraction c18ZipTip and MALDI-TOF/TOF

E18- Standard — BSA
E19- Blank – Background only
E20- Spot 1679 – no significant identification
E21- Spot 3447 – serum amyloid P-component--gi|148747488
E22- Spot 2840 – group specific component, isoform CRA_a--gi|149033741
E23- Spot 3379 – complement factor I--gi|149025945
E24- Spot 2248 – alpha-1-inhibitor III precursor--gi|554401
F1- Spot 932 – no significant identification
F2- Spot 3209 – serum amyloid P-component--gi|148747488
F3- Spot 2655 – serum amyloid P-component--gi|148747488
F4- Spot 808 – alpha-1-inhibitor III--gi|554402
F5- Spot 2780 – apolipoprotein C-III, isoform CRA_a --gi|149041554
F6- Spot 3769 – no significant identification

Date of completion: 031108 Spot set 020508_09161 Runs 26-27

Figure 32: Summary of up-regulated proteins identified in IgY pilot study of d-serine control (243/246) verses BEA 500 mg/kg (285/289).
Spots 13 - 23 listed

Woolard SS (DIGE) 2D gel—303/5 vs 320/4 18 spots pilot study



Date of completion: 031108

Spot set 020508_09161 Runs 5-6

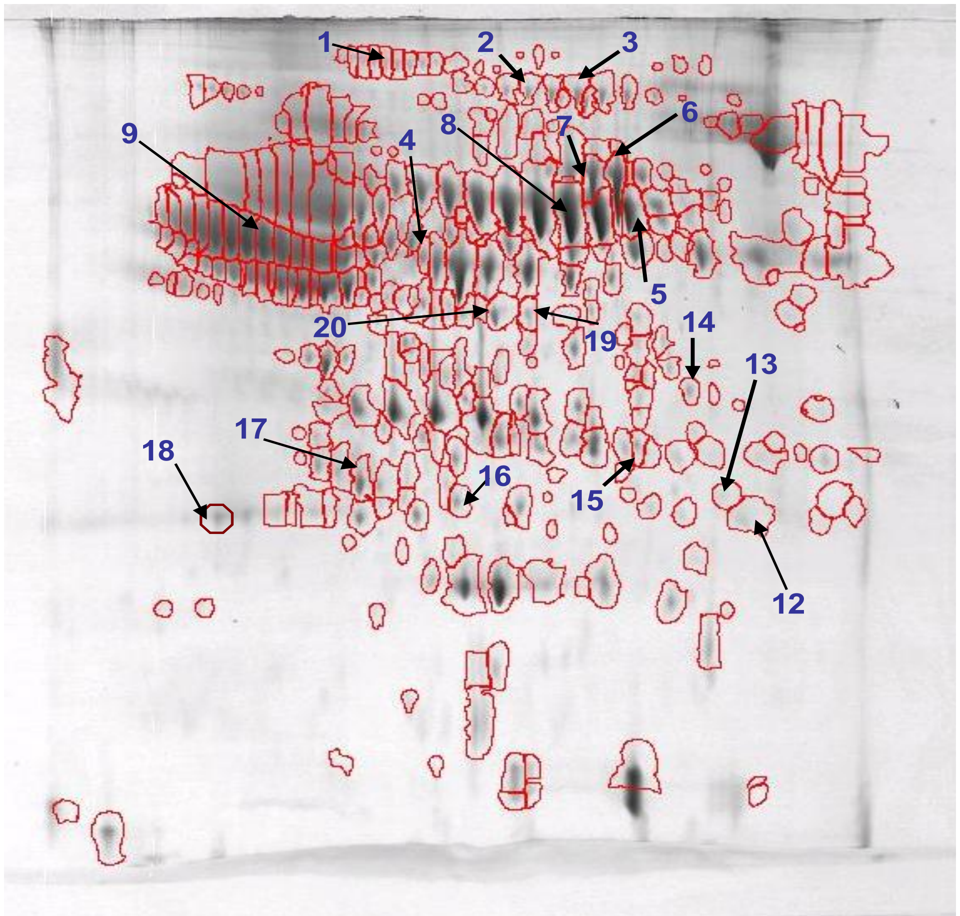
Summary of Protein Identification

Protein identification by in gel trypsin digestion, extraction c18ZipTip and MALDI-TOF/TOF

A22- Standard — BSA
A23- Blank – Background only
A24- Spot 1 – Inter-alpha-inhibitor H4 heavy chain--gi|2292988
B1- Spot 2 – Da1-24--gi|33086684
B2- Spot 3 – Da1-24--gi|33086684
B3- Spot 4 – Fibrinogen, gamma polypeptide--gi|61098186
B4- Spot 5 – Hemopexin precursor--gi|122065203
B5- Spot 6 – Albumin--gi|55391508
B6- Spot 7 – Albumin--gi|55391508
B7- Spot 8 – Hemopexin precursor--gi|122065203
B8- Spot 9 – Serine protease inhibitor A3L precursor (Serpin A3L) (Contrapsin-like protease inhibitor 3) (CPI-23)--gi|2507387 and thyroid hormone-regulated proteinase inhibitor--gi|92880 and Alpha-2-HS-glycoprotein precursor (Fetuin-A) (Glycoprotein PP63) (59 kDa bone sialic acid-containing)--gi|231468

Figure 33: Summary of proteins identified in IgY pilot study of puromycin control (303/305) versus puromycin 300 mg/kg (320/324). *Spots 1- 9 listed.*

Woolard SS (DIGE) 2D gel—303/5 vs 320/4 18 spots pilot study



Summary of Protein Identification

Protein identification by in gel trypsin digestion, extraction c18ZipTip and MALDI-TOF/TOF

B9- Spot 10 – Standard — BSA
B10- Spot 11 – Blank – Background only
B11- Spot 12 – Keratin contamination
B12- Spot 13 – rCG38388 or complement component 4a-- gi|149027977
B13- Spot 14 – Transferrin--gi|1854476
B14- Spot 15 – Pregnancy-zone protein or Alpha-1-macroglobulin precursor--gi|21955142 and Keratin contamination
B15- Spot 16 – Alpha-1-antiproteinase precursor--gi|112889
B16- Spot 17 – Apolipoprotein E precursor--gi|1703338
B17- Spot 18 – no significant identification
B18- Spot 19 – fibrinogen, gamma polypeptide--gi|61098186
B19- Spot 20 – Fibrinogen, gamma polypeptide--gi|61098186

Date of completion: 031108   Spot set 020508_09161 Runs 5-6

Figure 34: Summary of proteins identified in IgY pilot study of puromycin control (303/305) vs puromycin 300 mg/kg (320/324).
Spots 10 - 18 of 18 listed.

7.3 Discussion of Pilot Study Results

The Pilot Study was used to optimize the protocols necessary for a full study. However, this tentative study did not produce as much data on differential protein expression and subsequent identification as anticipated. Most of the proteins identified were generic glycoproteins, which act as carrier proteins, or typical serum abundant proteins such as albumin, transferrin, and fibrinogen. The proteins that *were* sequenced demonstrated the difficulties in serum immunodepletion and the pervasiveness of abundant carrier proteins throughout the bloodstream. The impediments in removing these proteins underscored the need for diligence during the initial serum enrichment utilizing the IgY column. One important element identified during protocol development was the need for enrichment of several aliquots of each sample twice. This redundancy was necessary to guarantee each aliquot was filtered as thoroughly as possible while ensuring protein concentrations high enough to stay above the DIGE gel lower limits of detection. The low sensitivity of the DIGE gels is an inherent weakness of the technique which limits the ability to find low abundant proteins. Because of the low sensitivity, the SameSpots software used to analyze the gels is only as accurate as the spot resolution in the gel as well as the user who refines the data. These limits make it crucial to have an experienced user defining and outlining the spots for the software to analyze. After optimizing the immunodepletion methodology and analyzing the Pilot Study results, the Full Study was initiated using the lessons learned.

8. FULL STUDY

8.1 Methods

8.1.1 Preparation of Samples for Full Study

The samples were prepared for pooling by organizing the individual serum samples into their respective drug and dose groups. The puromycin and BEA drug studies had 4 dose groups (control, low, medium, and high dose) with each group containing 5 animals, at least at the beginning of the studies. Serum samples were collected from each rat at three time points: pre-dose, 24 hours post dose, and 96 hours post dose (terminal sacrifice) to give a total of 15 individual samples for the puromycin and BEA studies. The 15 individual samples were condensed to create three pooled samples by combining the serum from all five rats according to time points.

Table 3 indicates the number and identification number of animals in each dose group and the time points collected from each rat. For puromycin and BEA, the charts below have an ―X” in a box to indicate that a serum sample was collected from the rat for that time point at that dose. If a rat died before the specific time point, such as with BEA - 500 mg/kg where all animals died before 24 hours, then the serum collected was labeled as ―Terminal Sacrifice”. Additionally, some bacterial contamination occurred during the animal study in a subset of BEA samples, so some samples were discarded entirely from the study. Because of the variation in number of subjects and time points among doses, the samples were pooled together using equal volumes from each subject so that a total volume of 75 μL was collected. This pooling was then split into 5 aliquots, each 15 μL in volume. The aliquots were then placed in the -20^{o} Celsius freezer for storage until they could be enriched with the ProteomeLab IgY spin column.

For D-serine, a second study was conducted with only three dose groups: a control-- 0 mg/kg, low dose--200 mg/kg, and high dose--500 mg/kg with two time points, 12 hours post dose and 24 hours post dose. The other difference between the D-serine study and the puromycin/BEA studies was the lack of an internal control for each rat. The puromycin and BEA studies collected three serum samples from each rat at a given

time point. The D-serine study sacrificed the rat subject at the designated time point to collect the serum sample. This method ensured enough volumes of serum were collected with the tradeoff being a lack of positive control from the animal itself. The D-serine study had no contamination issues or early deaths, so all pooled samples contained five rats from which 15 µL were combined and mixed to give a total volume of 75 µL. After mixing, the pooled sample was split into 5 aliquots of 15 µL and placed in a -20^{o} C freezer for storage.

8.1.2. Serum Immnunodepletion using IgY spin column

Specific samples (

Table 5A, 5B, 5C) were used from specific time points taken from each rat from puromycin and BEA Study 1 and D-serine Study 2 used in the Full Study. The data in

Table 5 demonstrates the need for sample pooling in this study and shows where animal study contaminations may have occurred as well as lethal doses for BEA. The Full Study samples were enriched using the IgY R-7 spin column and ProteomeLab protocol as described previously in the Pilot Study section with a few minor changes to optimize immunodepletion. One change was the addition of 0.1 mL of Protease Inhibitor cocktail to 9.9 mL of IgY Dilution buffer working solution to protect against protein degradation while working with samples at room temperature. The Dilution buffer plus protease inhibitor was used only for the FT fractions. The Wash fractions used regular Dilution buffer that was diluted from 10X to 1X per protocol.

Table 5: Individual animal samples used for Full Study Serum pooling.

A. Puromycin Animal Samples

Control Animal No.	Pre-Clinical	24 hr post dose	Terminal Sacrifice
301	x	x	x
302	x	x	x
303	x		x
304	x		x
305	x	x	x

75 mg/kg Animal No.	Pre-Clinical	24 hr post dose	Terminal Sacrifice
311	x	x	x
312	x	x	x
313	x	x	x
314	x	x	x
315	x	x	x

150 mg/kg Animal No.	Pre-Clinical	24 hr post dose	Terminal Sacrifice
316	x	x	x
317	x	x	x
318	x	x	x
319	x	x	x
320	x		x

300 mg/kg Animal No.	Pre-Clinical	24 hr post dose	Terminal Sacrifice
321	x	x	x
322	x	x	x
323	x	x	x
324	x	x	x
325	x		x

B. BEA Animal Samples

Control Animal No.	Pre-Clinical	24 hr post dose	Terminal Sacrifice
119	x		x
154	x	x	x
155	x	x	x
156	x		x

15 mg/kg Animal No.	Pre-Clinical	24 hr post dose	Terminal Sacrifice
139	x		x
140	x		x
141	x		x

50 mg/kg Animal No.	Pre-Clinical	24 hr post dose	Terminal Sacrifice
131	x		x
132	x	x	x
133	x		x
134	x	x	x
135	x	x	x

150 mg/kg Animal No.	Pre-Clinical	24 hr post dose	Terminal Sacrifice
118			x
128	x	x	x
129	x	x	x
130	x	x	x

500 mg/kg Animal No.	Pre-Clinical	24 hr post dose	Terminal Sacrifice
285			x
286			x
287			x
288			x
289			x

C. D-Serine Animal Samples

Dose	Time Point	Subject numbers			
Control	12 hr post dose	1	5	15	28
0 mg/kg	24 hr post dose	6	11	26	30
200 mg/kg	12 hr post dose	4	20	27	29
	24 hr post dose	2	12	19	24
500 mg/kg	12 hr post dose	7	8	17	22
	24 hr post dose	3	9	18	21

The other significant protocol change between the Pilot Study versus Full Study was the increased number of aliquots enriched. The individual Full Study samples were collected to give pooled samples containing 75 µL which were then divided into 5 aliquots of 15 µL each. The Full Study enriched 4 aliquots from each pooling creating a 1st Generation (1G) FT sample for each aliquot with a spare aliquot in case of error during the process. After enrichment, the FT and E fractions were concentrated to approximately 500 µL using the Amicon YM-3 spin cartridges. If a sample was concentrated to less than 500 µL, samples were diluted to the final volume of 500 µL and stored in a -20^{o} C freezer. Once all the 1G FT and E fractions were collected from each aliquot, NI protein assays and 1D gels were run to monitor the column efficiency. After the assays, the 1G FT samples from each aliquot were individually enriched a second time using the same protocol as before. The 2nd Generation (2G) FT and E fractions were collected and concentrated using Amicon YM-3 spin cartridges. ProteomeLab lists the effective life of the IgY spin columns as 100 runs but column degradation was noticeable when centrifugation required higher speeds, 6000 – 7000 x g, and at longer spin times, 1 – 2 minutes. Once the paper filter in the plastic column was irrevocably degraded, the protocol described the transfer of the resin beads to a spare plastic column housing by mixing the beads in 500 µL of dilution buffer and pipetting the slurry into a spare column. The IgY kit came with two columns and six spare plastic housings. Once all the spare column housings were used, the column was retired from use. In all, 7 columns were required to enrich all the aliquots of the pooled samples twice.

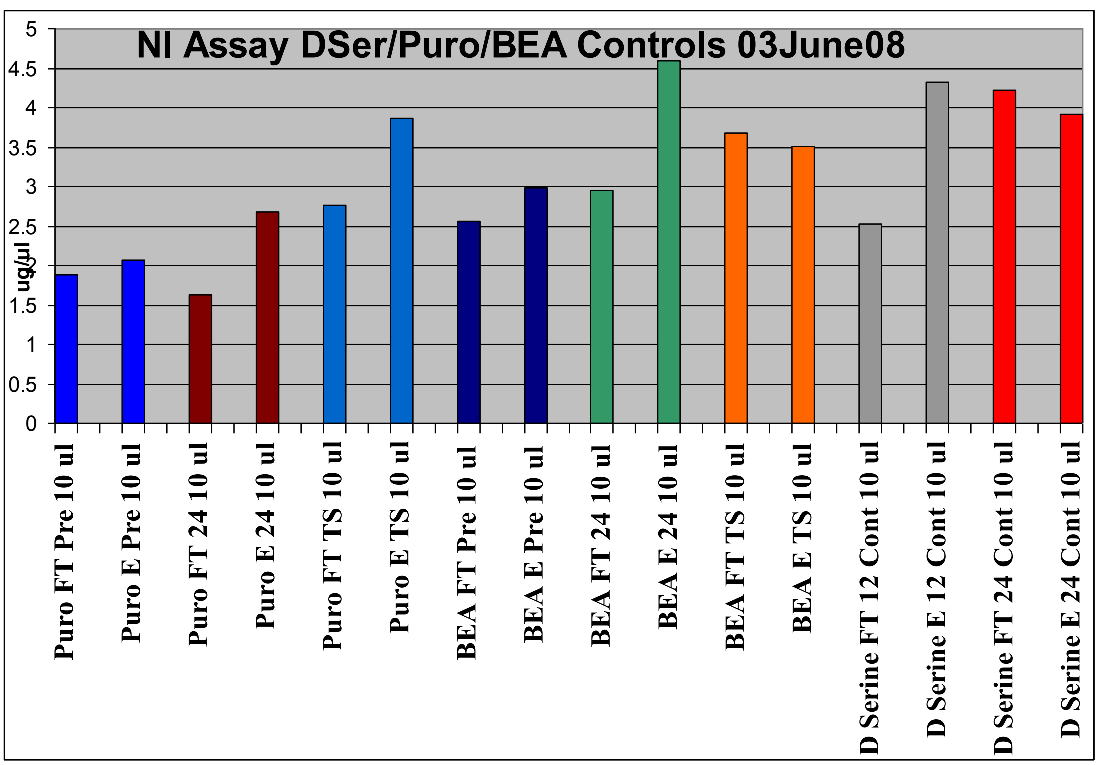
8.2 Full Study Results

After all the 1G aliquots were concentrated, the buffer was exchanged in the Microcon tubes by adding 4 mL of 2M urea, centrifuged for 70 min at 4000 rpm at 4^{o} C using a Thermo Centra CLR with a Thermo 243 rotor. A total of 3 buffer exchanges were performed with samples concentrated to a final volume between 200 - 400 ul. NI protein assays and small, 2D PAGE gels were run on the 2G samples to confirm the final enrichment and to measure the concentrations for the large DIGE gels.

8.2.1 Protein Assays on 1G Samples

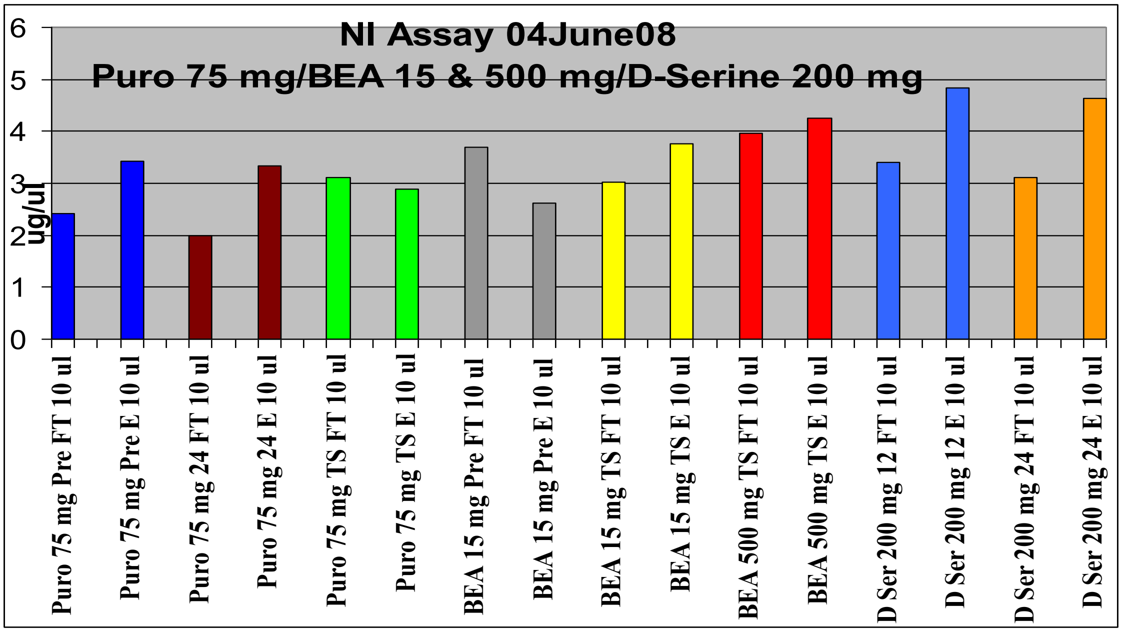
The protein assays (Figure 35 A-D) demonstrate that immunodepletion by the IgY columns was successful in two ways. First, the high protein concentration values across all samples. All samples had at least 1 μg/μl of protein with many in the 2 – 4 μg/μl range which indicated there would be enough protein for the DIGE gels after further enrichment. The second positive characteristic was the higher concentration value for the Elution fractions. The higher value for the Elution fractions indicated that the IgY column removed most of the high abundant proteins meaning greater resolution on the 2D gels. After the protein concentrations were measured, the samples were run on small 1D SDS-PAGE gels in order to visualize the amount of abundant proteins that were removed.

A



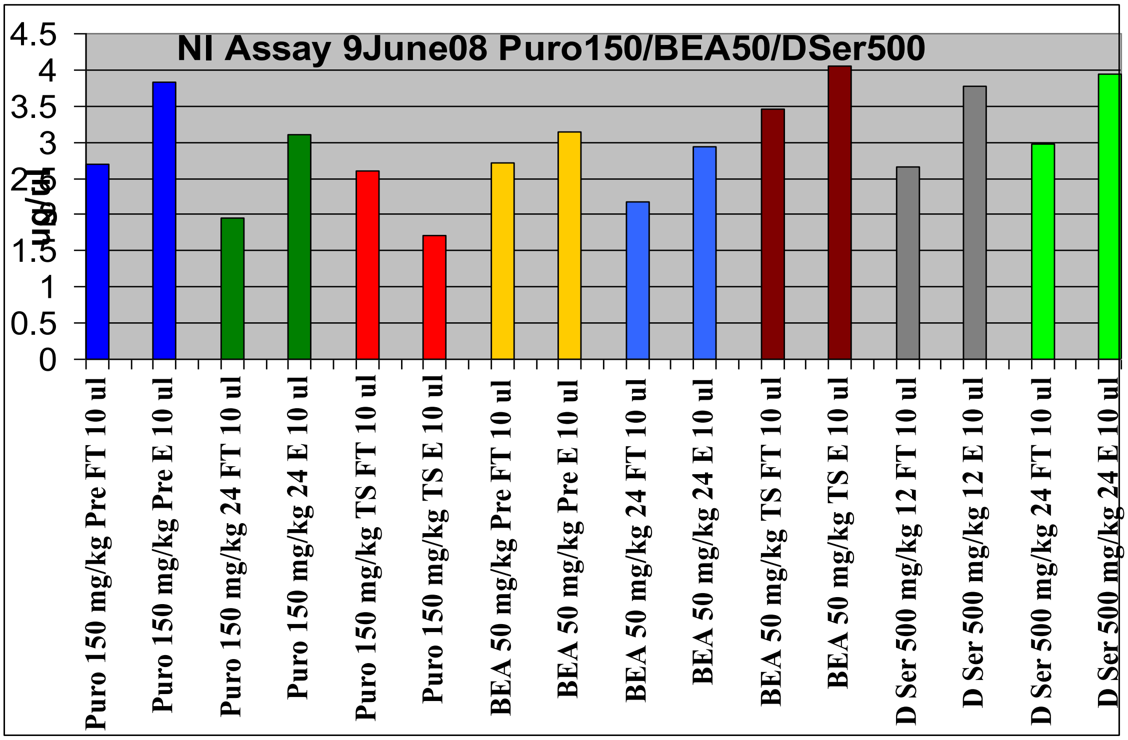
.

B



C

Distribution A: Approved for Public release; distribution unlimited.


NI Assay 9June08 Puro150/BEA50/DSer500
ug/ul
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Puro 150 mg/kg Pre FT 10 ul
Puro 150 mg/kg Pre E 10 ul
Puro 150 mg/kg 24 FT 10 ul
Puro 150 mg/kg 24 E 10 ul
Puro 150 mg/kg TS FT 10 ul
Puro 150 mg/kg TS E 10 ul
BEA 50 mg/kg Pre FT 10 ul
BEA 50 mg/kg Pre E 10 ul
BEA 50 mg/kg 24 FT 10 ul
BEA 50 mg/kg 24 E 10 ul
BEA 50 mg/kg TS FT 10 ul
BEA 50 mg/kg TS E 10 ul
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D Ser 500 mg/kg 24 E 10 ul

D

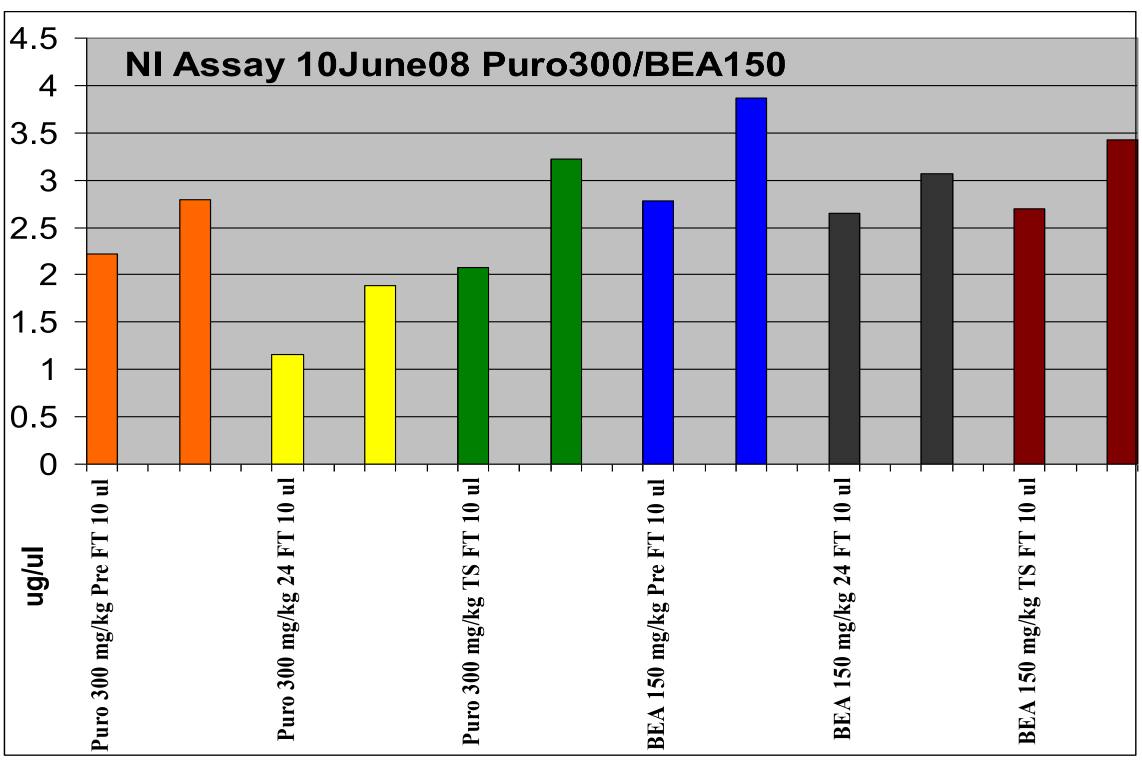

NI Assay 10June08 Puro300/BEA150
ug/ul
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Puro 300 mg/kg Pre FT 10 ul
Puro 300 mg/kg 24 FT 10 ul
Puro 300 mg/kg TS FT 10 ul
BEA 150 mg/kg Pre FT 10 ul
BEA 150 mg/kg 24 FT 10 ul
BEA 150 mg/kg TS FT 10 ul

Figure 35: Non-interfering Protein Assays of Full Study Samples used to monitor column effectiveness and values for use in 2D DIGE gels.

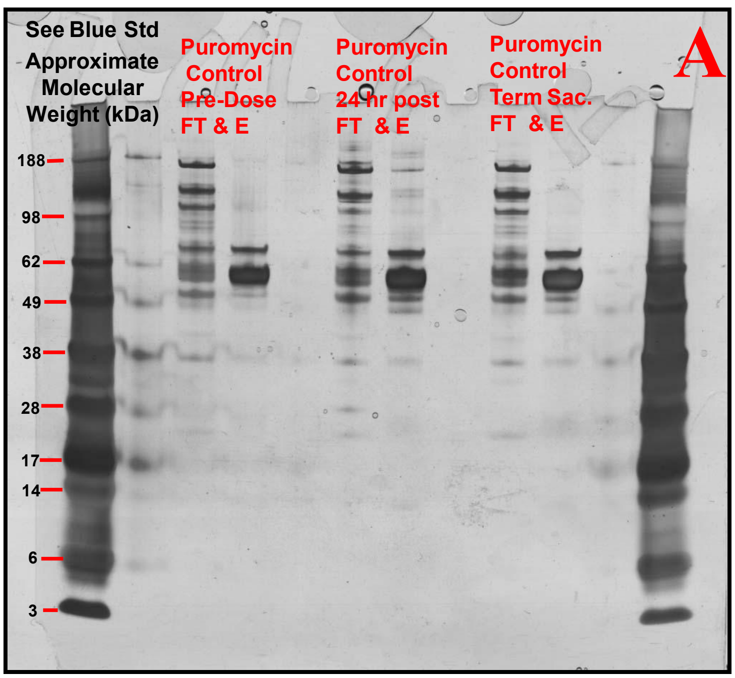
A) *Samples:*			
	Puromycin	*Control samples*	*Pre dose/24 hour post dose/Terminal Sac*
	BEA	*Control samples*	*Pre dose/24 hour post dose/TerminalSac*
	D- Serine	*0 mg/kg*	*12 and 24 hours*
B) *Samples:*			
	Puromycin	*75 mg/kg*	*Pre dose/24 hour post dose/Terminal Sac*
	BEA	*15 & 500 mg/kg*	*Pre dose/TS (15 mg/kg) and TS (500 mg/kg)*
	D- Serine	*200 mg/kg*	*12 and 24 hours*
C) *Samples:*			
	Puromycin	*150 mg/kg*	*Pre dose/24 hour post dose/Terminal Sac*
	BEA	*50 mg/kg*	*Pre dose/TS (15 mg/kg) and TS (500 mg/kg)*
	D- Serine	*500 mg/kg*	*12 and 24 hours*
D) *Samples:*			
	Puromycin	*300 mg/kg*	*Pre dose/24 hour post dose/Terminal Sac*
	BEA	*150 mg/kg*	*Pre dose/TS (15 mg/kg) and TS (500 mg/kg)*

8.2.2 1st Generation Samples 1D SDS-PAGE gels

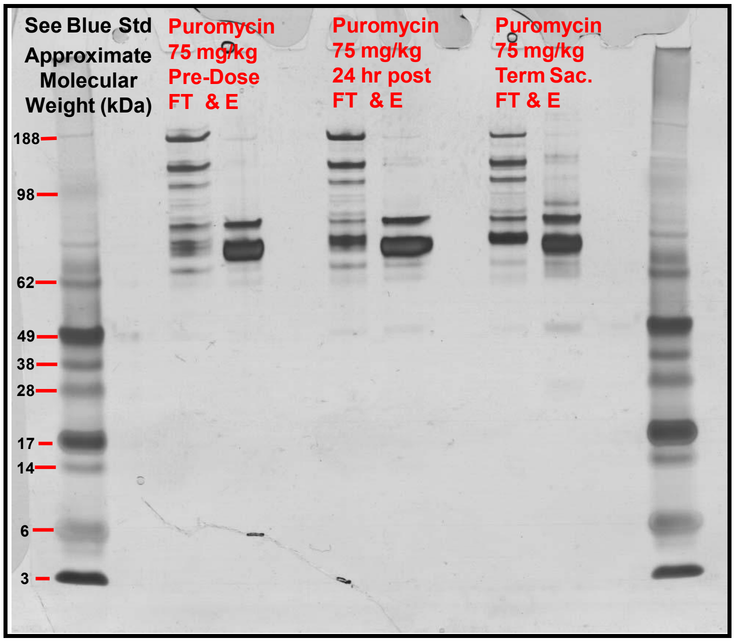
All 1D gels were silver stained with Sigma's Proteosilver silver stain kit which can identify proteins in concentrations as small as 0.1 ng/µL. Given this high degree of sensitivity, all samples were diluted 1:10 with Millipore water to reach a final concentration between 100 and 400 ng/µl and a final volume of 10 ul. 10 ul of SeeBlue Plus 2 marker was placed in wells 1 and 12 of each gel and 10 ul of each sample was placed in FT & E pairs. Gels were run according to Invitrogen protocols using 3-(N-morpholino)propanesulfonic acid (MOPS) buffer at 120 V for approximately 1 hour 20 minutes. Gels were immediately stained according to Sigma's ProteoSilver Silver Stain kit protocol. Afterwards, the gels were scanned using LabScan v 5.0 software on an Amersham Image Scanner. All 1D gels were purchased from Invitrogen using the 4-12% gradient gels 1mm x 12 well, Catalog number NP0322BOX, Lot numbers 6071173 and 7120610. Invitrogen SeeBlue Plus 2 Prestained Standard was used as the marker for each gel (Figure 36 A-J.)

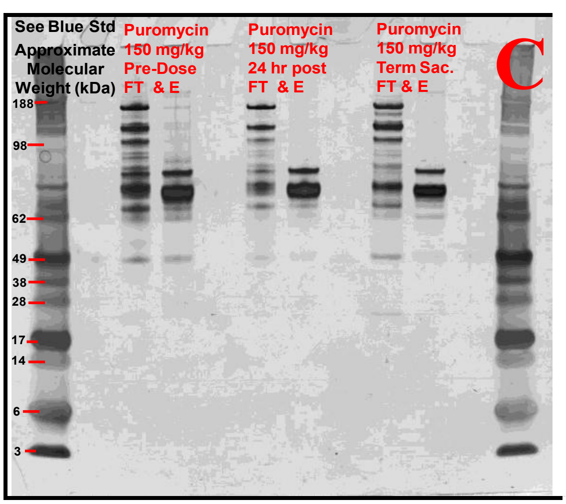
As a reference, listed below are the seven proteins targeted for removal by the IgY column and their respective molecular weights. Immediately visible in the E fraction wells is the large albumin band at 68 kDa which shows up much darker in the E fraction compared to the FT fraction.

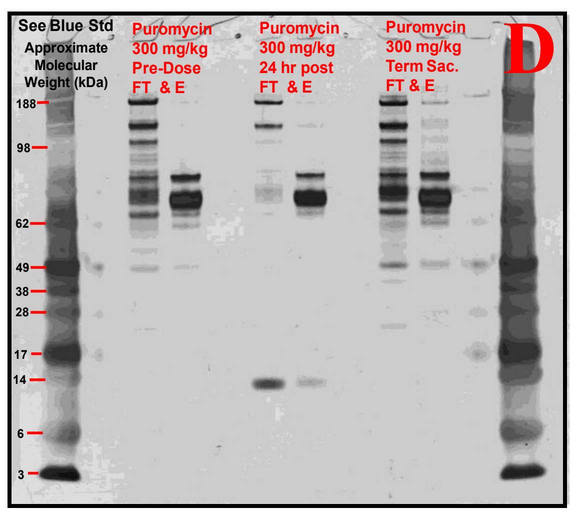
Rat Albumin	68,731 Da	Rat IgM	70,000 Da
Rat IgG	146,000 Da	Rat alpha1-antitrypsin	52,000 Da
Rat Transferrin	76,500 Da	Rat Haptoglobin	95,000 Da
Rat Fibrinogen	340,000 Da		



B


See Blue Std
Approximate
Molecular
Weight (kDa)
Puromycin
75 mg/kg
Pre-Dose
FT & E
Puromycin
75 mg/kg
24 hr post
FT & E
Puromycin
75 mg/kg
Term Sac.
FT & E
188
98
62
49
38
28
17
14
6
3


See Blue Std
Approximate
Molecular
Weight (kDa)
Puromycin
150 mg/kg
Pre-Dose
FT & E
Puromycin
150 mg/kg
24 hr post
FT & E
Puromycin
150 mg/kg
Term Sac.
FT & E
C
188
98
62
49
38
28
17
14
6
3


See Blue Std
Approximate
Molecular
Weight (kDa)
Puromycin
300 mg/kg
Pre-Dose
FT & E
Puromycin
300 mg/kg
24 hr post
FT & E
Puromycin
300 mg/kg
Term Sac.
FT & E
D
188
98
62
49
38
28
17
14
6
3

E

F

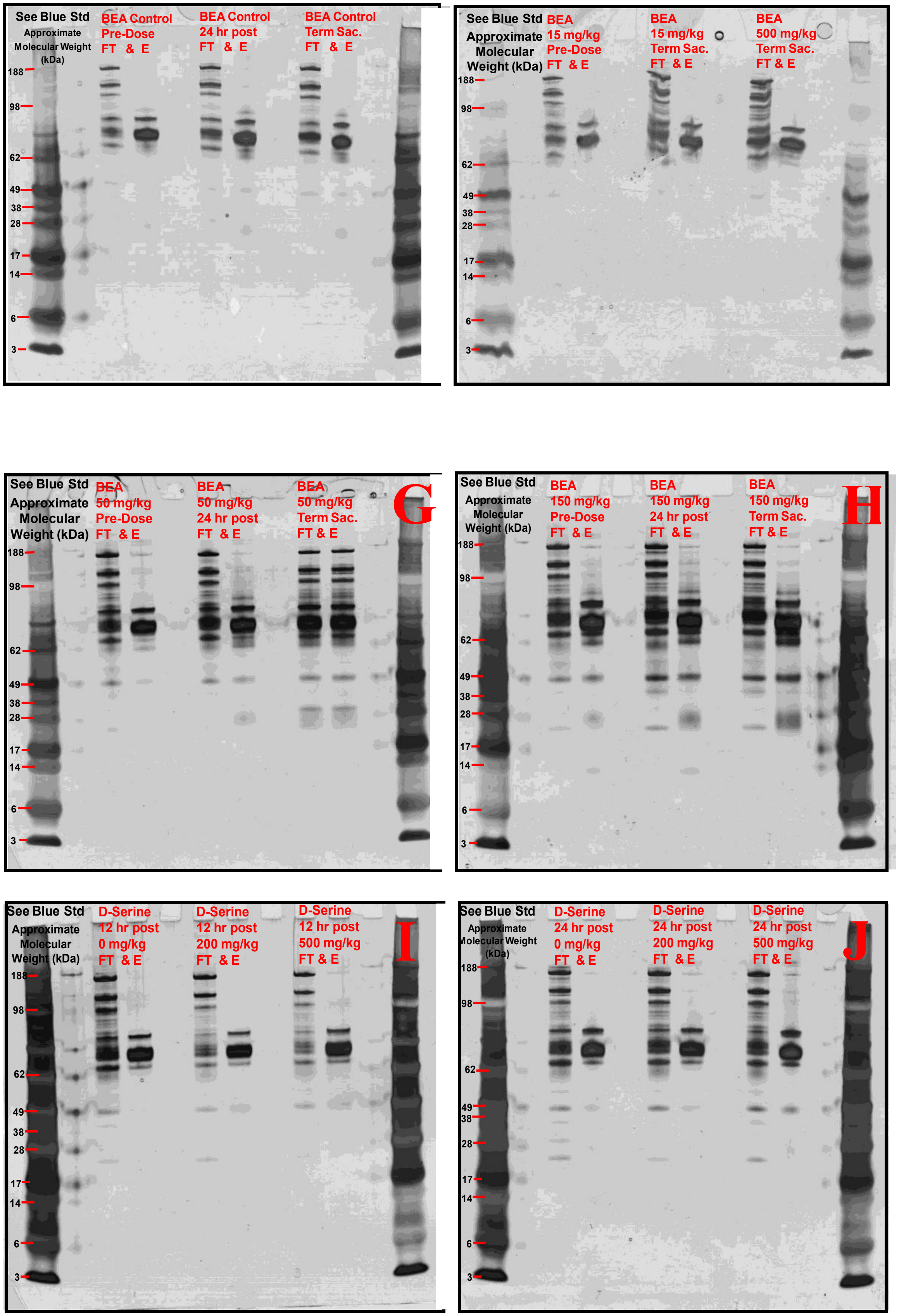

See Blue Std
Approximate Molecular Weight (kDa)
188
98
62
49
38
28
17
14
6
3
BEA Control Pre-Dose FT & E
BEA Control 24 hr post FT & E
BEA Control Term Sac. FT & E
See Blue Std
Approximate Molecular Weight (kDa)
BEA 15 mg/kg Pre-Dose FT & E
BEA 15 mg/kg Term Sac. FT & E
BEA 500 mg/kg Term Sac. FT & E
See Blue Std
Approximate Molecular Weight (kDa)
BEA 50 mg/kg Pre-Dose FT & E
BEA 50 mg/kg 24 hr post FT & E
BEA 50 mg/kg Term Sac. FT & E
G
See Blue Std
Approximate Molecular Weight (kDa)
BEA 150 mg/kg Pre-Dose FT & E
BEA 150 mg/kg 24 hr post FT & E
BEA 150 mg/kg Term Sac. FT & E
H
See Blue Std
Approximate Molecular Weight (kDa)
D-Serine 12 hr post 0 mg/kg FT & E
D-Serine 12 hr post 200 mg/kg FT & E
D-Serine 12 hr post 500 mg/kg FT & E
I
See Blue Std
Approximate Molecular Weight (kDa)
D-Serine 24 hr post 0 mg/kg FT & E
D-Serine 24 hr post 200 mg/kg FT & E
D-Serine 24 hr post 500 mg/kg FT & E
J

Figure 36: 1D SDS-PAGE gels of 1st Generation (1G) FT & E fractions after IgY column enrichment.

Gels were used to monitor over abundant protein removal from pooled samples for use in full study. Images are as follows: ***A)*** *Puromycin Controls;* ***B)*** *Puromycin 75 mg/kg dose;* ***C)*** *Puromycin 150 mg/kg;* ***D)*** *Puromycin 300 mg/kg;* ***E)*** *BEA Controls;* ***F)*** *BEA 15 mg/kg & 500 mg/kg;* ***G)*** *BEA 50 mg/kg;* ***H)*** *BEA 150 mg/kg;* ***I)*** *D-Serine 12 hr post dose;* ***J)*** *D-Serine 24 hr post dose*

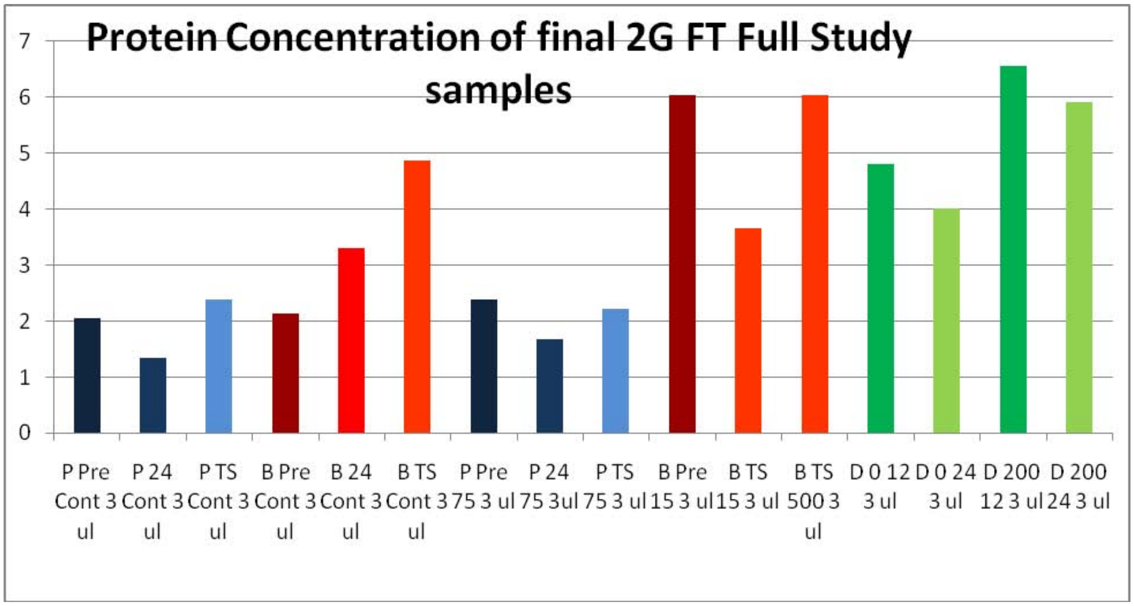
8.2.3 2nd Generation FT fraction NI Protein Assay and small 2D gels

The protein concentration measurements of the final, condensed samples were used for calculating the volume needed to run the DIGE gels. The assay indicated potential problems with the puromycin 24 hour 150 mg/kg and 300 mg/kg samples because of low concentration values (Figure 37). However, a quick calculation showed that the volumes of samples needed for those two specific DIGE gels would not exceed the total 350 µL limit. Based on those calculations and time constraints, it was deemed appropriate to continue with the study. After the NI assays, two FT fraction samples were run on a small, 2D gel as a final quality control measure to ensure that most overabundant proteins were removed from the serum samples. The samples tested were:

Gel 1:	D-Serine	0 mg/kg	24 hr post dose
Gel 2:	Puromycin	300 mg/kg	Terminal Sacrifice

In order to run the 2D gels, the two samples were prepared for separation in the first dimension based on isoelectric point. First, 200 ml of Rehydration Buffer was generated using GRI protocols. A total of 23.14 mg DTT was added to the buffer, vortexed, and centrifuged to dissolve all particulates. Next, the Rehydration buffer was sonicated for 1 minute then placed on an end-to-end rotator for 15 minutes. This protocol was repeated three times for a total of four sonication/mixing cycles. Next, samples were pipetted into IPG trays and IPG strips (Immobiline Dry Strip pH 4 – 7, 7 cm from GE, 17-6001-10, lot # 10 009958) were placed into the tray wells and the ends were covered. The samples were allowed to re-swell overnight. After all protein samples had been fully absorbed by the IPG strip, the samples were run in the first dimension by placing the gel tray in a 1D gel box (zoom IPG) a new mod panel was added and filled with ultra-pure

water. The cover was put into place and a gradient voltage was applied in the following cycle: 0 -175 V for 15 minutes, 175 – 2000 V for 45 minutes, and at 2000 V for 1 hour 45 minutes.



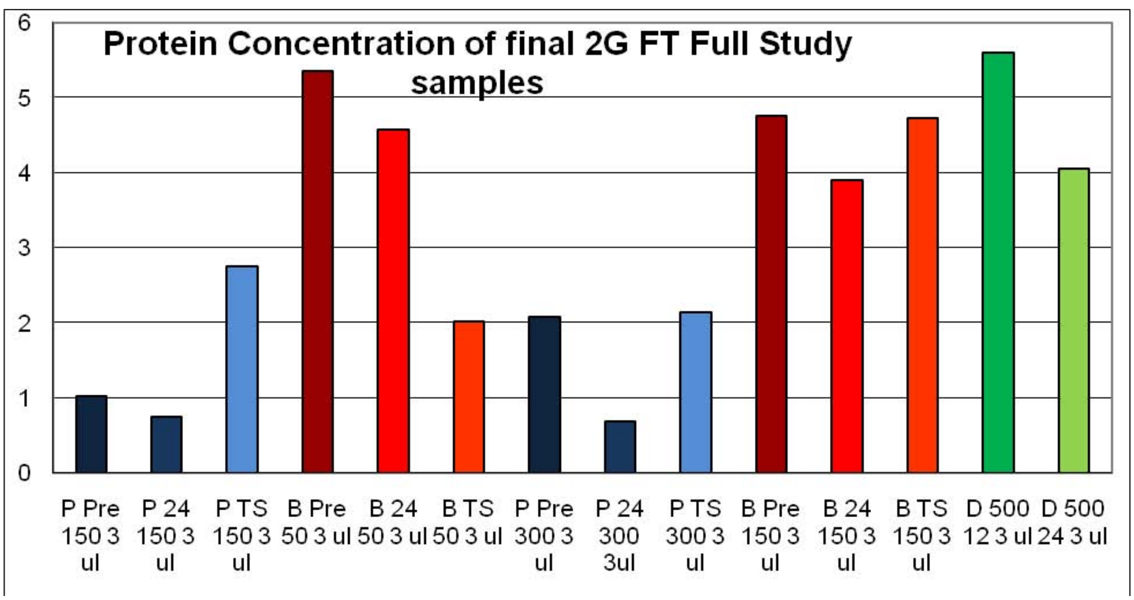
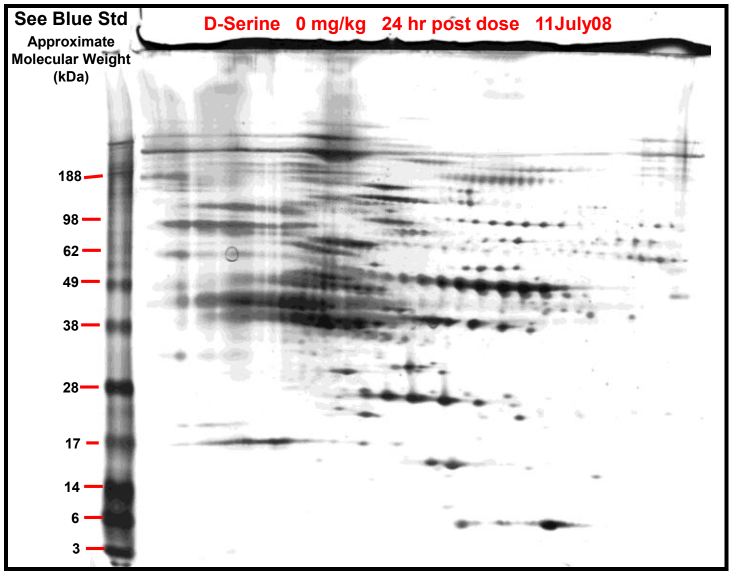


Figure 37: NI Protein Assays measuring the final protein concentrations of the condensed, buffer exchanged 2G FT fractions of the Full Study samples.

Results show IgY column still removing over abundant proteins and the values then used to calculate sample volumes needed for 2D DIGE gels.P = puromycin, B = BEA, D = D-serine, Pre = predose, 24 = 24 hour post-dose, TS = terminal sacrifice

After the samples were separated in the first dimension, the IPG strips were removed and placed in the well of a small 2D gel cassette from Invitrogen. A total of 10 uL of SeeBlue Plus 2 ul prestained standard was added to a separate well and the two gels were run at 110V for 1 hour 20 minutes. After separation in the second dimension, the gels were silver stained using the Proteolab SilverStain kit using the protocol included in the kit. After staining, the gels were placed on an Amersham scanner and scanned into the computer using LabScan software (Figure 38).

A



B

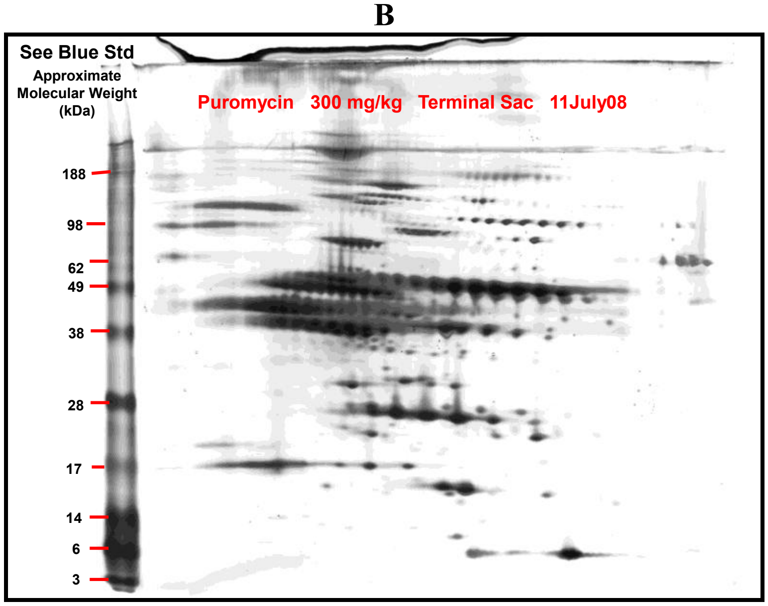


Figure 38: Small, 2D SDS-PAGE gels of 2nd Generation FT samples.

The gels were a final quality control measure to ensure that over abundant proteins were removed and that adequate resolution of low abundant proteins would permit spot analysis. The images are listed as (A) D-Serine 0 mg/kg 24 hr small 2D gel; (B) Puromycin 300 mg/kg Terminal Sacrifice small 2D gel

These small gels demonstrated a significant increase in protein resolution and over-abundant protein removal compared to the original gel tests of the Agilent MARS

column. Based on the clarity of the results it was determined that the resolution was high enough to warrant continuation with the project and preparation for the large DIGE gels.

8.3 2D DIGE Methods and Results

Once the small 2D gels confirmed the removal of abundant proteins from the samples, the large 2D DIGE gels were begun. All work, such as sample labeling with the fluorescent CyDyes, the separation in the first dimension, separation in the second dimension, fluorescent scanning, silver staining, and finally regular scanning of the silver stained gels, was done according to GRI protocols as described in earlier sections (Figure 39). The sample list set up for the gels is listed below in Table 6.

Table 6: Listing of the pairwise comparisons in each individual gel.

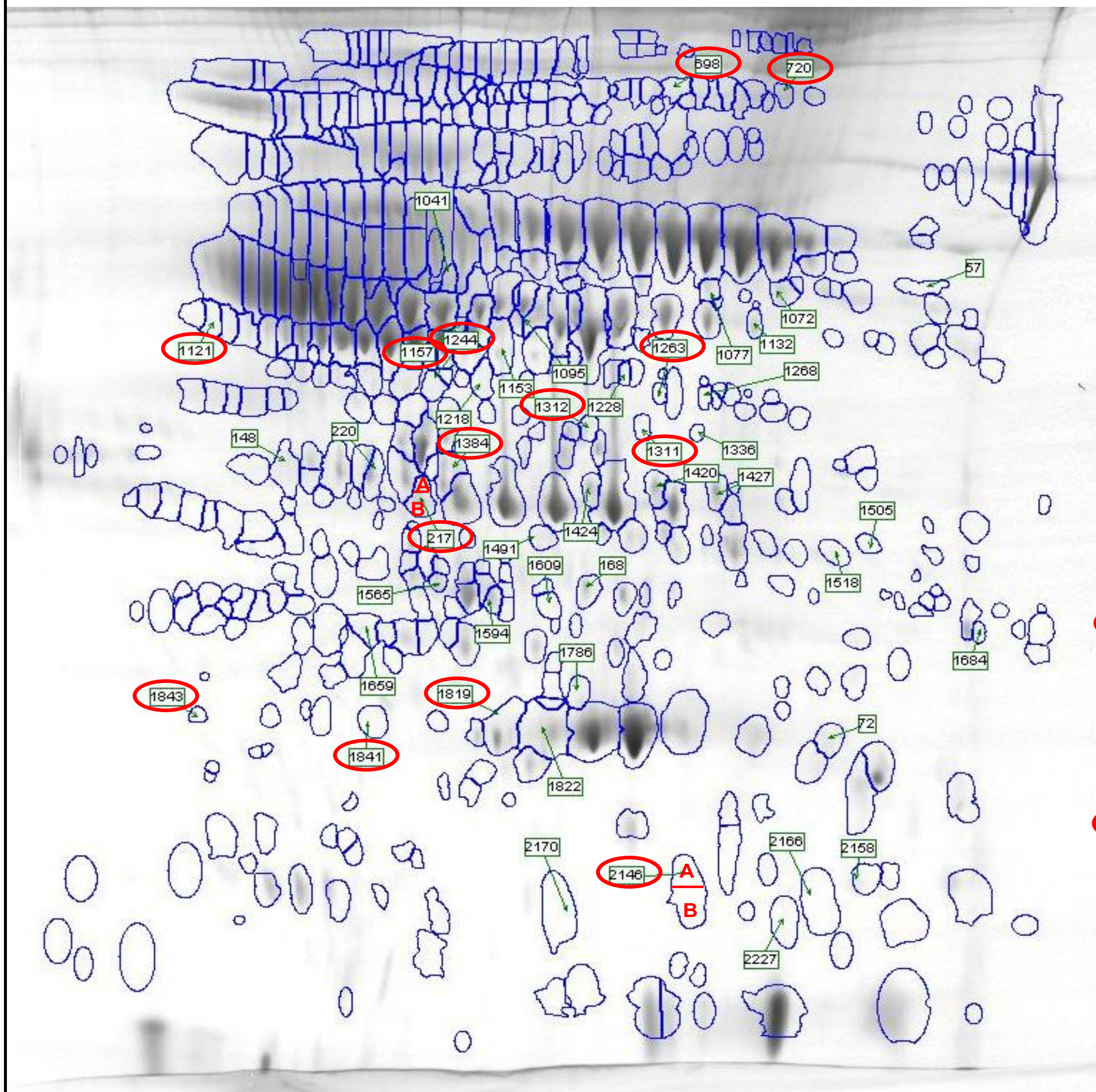
P – Pre dose time point, 24 – 24 hours post dose time point, TS – Terminal Sacrifice time point.

	Puromycin	
Pairwise comparisons		
75 mg/kg	gel 1	control P vs 75 (P)
	gel 2	control 24 vs 75 (24)
	gel 3	control TS vs 75 (TS)
150 mg/kg	gel 4	control P vs 150 (P)
	gel 5	contol 24 vs 150 (24)
	gel 6	control TS vs 150 (TS)
300 mg/kg	gel 7	Control P vs 300 (P)
	gel 8	Control 24 vs 300 (24)
	gel 9	Control TS vs 300 (TS)
	BEA	
15 mg/kg	gel 10	Control P vs 15 (P)
	gel 11	Control TS vs 15 (TS
50 mg/kg	gel 12	Control P vs 50 (P)
	gel 13	Control 24 vs 50 (24)
	gel 14	Control TS vs 50 (TS)
150 mg/kg	gel 15	Control P vs 150 (P)
	gel 16	Control 24 vs 150 (24)
	gel 17	Control TS vs 150 (TS)
500 mg/kg	gel 18	Control TS vs 500 (TS)

	D-Serine	
200mg	gel 19	Control 12 vs 200 (12)
	gel 20	Control 24 vs 200 (24)
500mg	gel 21	Control 12 vs 500 (12)
	gel 22	Control 24 vs 500 (24)

A

Woolard 2D SS-DIGE gels



Summary of Protein Identification

Protein identification by in gel trypsin digestion, extraction c18ZipTip and MALDI-TOF/TOF

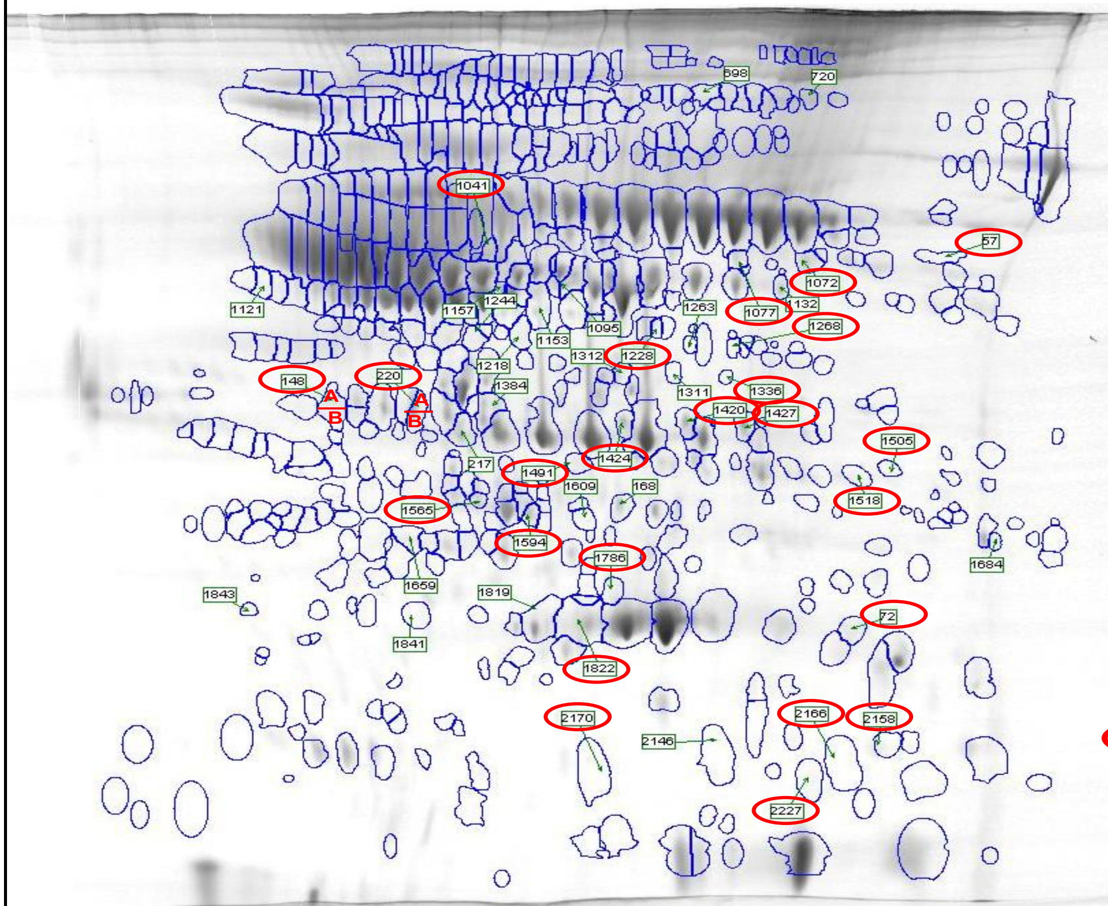
M9- Standard -- BSA
M10- Blank – Background only
M11- D 698 – Complement Factor B--gi|47059181
M12- D 720 – Complement Factor B--gi|47059181
M13- D 1384 – Murinoglobulin 1 homolog--gi|12831225
M14- D 1843 – No significant identification
M15- D 1841 – Pregnancy-zone protein--gi|21955142
M16- D 1819 – Serine protease inhibitor 2b--gi|6981576
M17- D 2146 A – No significant identification
M18- D 2146 B – No significant identification
N3- BSA
N4- P 1544 – Apolipoprotein E--gi|37805241
N5- P 1157 – Angiotensinogen--gi|19705570
N6- P 217 A – Pregnancy-zone protein--gi|21955142
N7- P 217 B – Pregnancy-zone protein--gi|21955142
N8- P 1122 – Fetuin Beta--gi|17865327
N9- P 1244 – Keratin Contamination
N10- P 1263 A – Fetuin Beta--gi|17865327
N11- P 1263 B – Keratin Contamination
N12- P 1311 A – Fetuin Beta-- gi|17865327
N13- P 1311 B – No significant identification
N14- P 1121 – Serine Protease Inhibitor 2a--gi|32563565
N15- P 1312 – No significant identification

Date of completion: 1029808

Spot set 092508_09173; Runs 11-14, 16-17

B

Woolard 2D SS-DIGE gels



N17- P 1041 – 1st ID) Serine (or cysteine) peptidase inhibitor, clade C (antithrombin), member 1--gi|58865630; **2nd ID) Inter-alpha-inhibitor H4 heavy chain--gi|2292988–tentative**

N18- P 1077 – Apolipoprotein H--gi|57528174

N19- P 2158 – No significant identification

N20- D 72 – **Hornerin--gi|57864582—tentative**

N21- D 57 – Apolipoprotein H--gi|57528174

N22- P 1822 – Apolipoprotein A-I--gi|2145145

O1- D 2227 – **Alpha-1 proteinase inhibitor 3--gi|609246--tentative**

O2- D 2166 – Alpha-1-antiproteinase precursor--gi|112889

O6- P 1268 – Fetuin Beta--gi|17865327

O7- P 1228 – Fibrinogen, gamma polypeptide--gi|61098186

O8- P 148 A – No significant identification

O9- P 148 B – No significant identification

O10- P 220 A – **Serine protease inhibitor 2b--gi|6981576--tentative**

O11- P 220B – Keratin contamination

O12- P 1786 – Inter-alpha-inhibitor H4 heavy chain--gi|2292988

O13- P 1072 – Apolipoprotein H-- gi|57528174

O14- P 1336 – No significant identification

A4- P 1859 – Glutathione peroxidase--gi|1586514

A5- P 2170 – Alpha2u globulin--gi|21954404

A6- P 1491 – No significant identification

A7- P 1424 – Pregnancy-zone protein--gi|21955142

A8- P 1427 A – Pregnancy-zone protein--gi|21955142

A9- P 1427 B – No significant identification

A10- P 1505 – No significant identification

A11- P 1594 – Apolipoprotein E--gi|37805241

A12- P 1565 – Apolipoprotein E--gi|37805241

A13- P 1518 – No significant identification

A14- P 1420 – Pregnancy-zone protein-- gi|21955142

Proteins listed in **bold red are tentative protein identifications** indicating that the protein is likely present, but it is recommended that this protein be confirmed prior to additional experimentation based solely on this MS identification

Date of completion: 102908

Spot set 102308_09161; Runs 2-8

Figure 39: Result summary of the final reference gel showing the location of the protein spots targeted as potential biomarkers and their subsequent identifications using MALDI-TOF/TOF mass spectrometry and MASCOT database searching.

Image (A) 20 protein spots and their identification (B) 28 protein spots and their identification

8.4 Protein Spot Selection Method and Results

The PG240 program was used to manipulate all data once imported into the program. First, the BEA 150 mg/kg Pre-Dose gel was designated the main reference gel. Next, the proteins spots from each gel were sorted by increased spot intensity based on a comparison of the Cy 5 labeled post-dose sample versus the Cy 3 labeled Pre-dose control (Figure 39). Thus, all gels act as single-point statistics with the spot intensity values based on a relative scale between the Cy 5 vs. Cy 3 dyes for each individual gel only (Table 7). This lack of statistics could not be avoided since the samples had to be pooled due to time and money constraints. Once the spots were organized based on intensities, a cut-off value of 1.5 fold increase was set and all spots with relative increases of 1.5-fold or greater were cut and pasted into Excel spreadsheets. The number of protein spots varied from each gel. In some gels, 25 spots were identified while others listed over 300 spots. In order to quickly cull through all the spots and identify one spot listed in all gels, all the protein spots were grouped based on dose- and time-points and relative intensities for each drug.

Table 7: Sample charts of protein spots and their relative increases versus control samples for three gels.

Charts demonstrate the typical data set used for analysis when searching for dose- and time-dependent toxicity trends from the full study. Data imported from the PG240 SameSpots software.

Ref. Spot #	Cy 3 Control Rat, Terminal Sac, 75 mg/kg	Cy 5 Dosed Rat, Terminal Sac, 75 mg/kg
Ref. Spot #	**Reference Volume of Protein Spot**	**Relative Volume X-fold increase**
2	1	2.084
12	1	2.231
20	1	3.14
40	1	2.983
44	1	2.029
45	1	2.468
51	1	4.343
56	1	2.01
72	1	2.165

Ref. Spot #	Cy 3 Control Rat, Terminal Sac, 150 mg/kg	Cy 5 Dosed Rat, Terminal Sac, 150mg/kg
Ref. Spot #	**Reference Volume of Protein Spot**	**Relative Volume X-fold increase**
2	1	1.893
21	1	1.8
37	1	1.569
38	1	4.496
40	1	3.016
44	1	2.287
48	1	2.082
53	1	4.716
65	1	1.909
72	1	1.988

Ref. Spot #	Cy 3 Control Rat, Terminal Sac, 300 mg/kg	Cy 5 Dosed Rat, Terminal Sac, 300 mg/kg
Ref. Spot #	**Reference Volume of Protein Spot**	**Relative Volume X-fold increase**
1	1	1.582
2	1	2.382
3	1	2.008
4	1	2.588
5	1	3.143
8	1	1.536
10	1	2.42
12	1	2.777
13	1	3.7
14	1	2.652

A histogram was made to see which protein spots indicated increases in all three gels for each dose or time point (Figure 40). Once spots were picked to represent a series of time points or doses from the histogram, those spots were combined into a larger pool to identify spots across all time points and doses (Table 8). The ultimate goal was to identify as many protein spots as possible that showed an increase in all 22 gels. The spots that showed up in the most gels and had the greatest average differences in intensities were picked for MS identification.

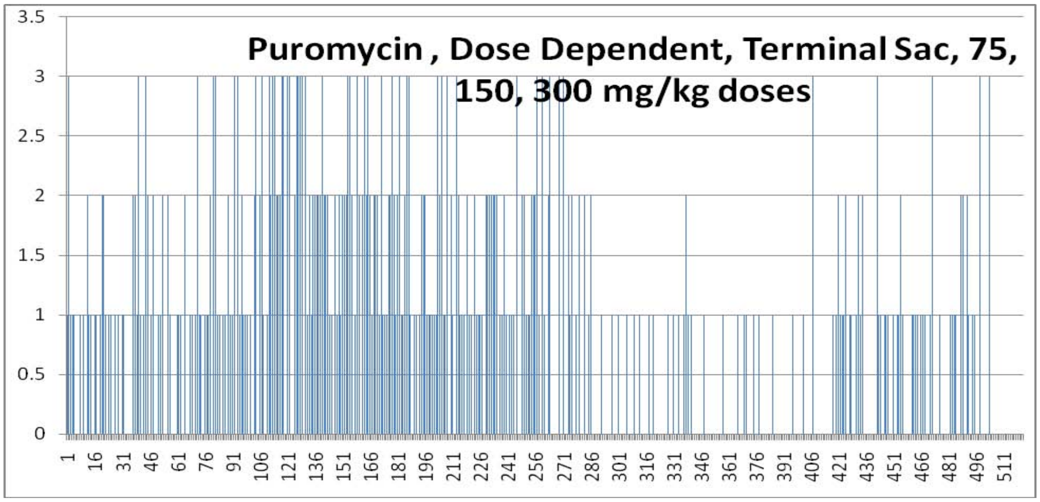


Figure 40: Histogram of protein spots in the three doses of the puromycin, Terminal Sacrifice time point gels.

The histogram showed which protein spots were up-regulated in multiple gels. The spots identified represented the Terminal Sac time point in the dose-dependent search. The spots listed in all three gels were combined with other histogram results to make the final spot selection.

Table 8: Sample chart demonstrating the relative volumes between the diseased serum samples versus the control samples for protein spot tentatively identified as hornerin.

Ref. Spot	**Control TS vs 75 TS Cy3**	**Control TS vs 75 TS Cy5**		**Ref. Spot**	**Control TS vs 150 TS Cy3**	**Control TS vs 150 TS Cy5**		**Ref. Spot**	**Control TS vs 300 TS Cy3**	**Control TS vs 300 TS Cy5**
Ref. Spot	Volume	Volume		Ref. Spot	Volume	Volume		Ref. Spot	Volume	Volume
72	1	2.165		72	1	1.988		72	1	3.907

9. CONCLUSIONS

The final results of the DIGE study identified two proteins of interest, glutathione peroxidase and hornerin, which may be validated as biomarkers of sub-clinical renal injury by additional work. Of the 48 protein spots tagged by the ‗SameSpots' software, 13 spots had protein concentrations lower than the detection limit of the MALDI thus no identification could be made. Three of the 48 spots were contaminated with keratin so no identification was possible. Of the remaining 32 spots, 7 were apolipoproteins which are found abundantly throughout serum and 4 spots were identified as fetuin β, a glycoprotein which is a member of the cystatin superfamily found in abundance throughout the body.

Other common proteins identified were 3 serine protease inhibitors and one α-1 proteinase inhibitor which are proteins designed to deactivate serine proteases. Serine protease is a generic name that applies to enzymes which catalyze the hydrolysis of peptide bonds through a nucleophilic attack by a serine active site. The prevalence of such inhibitors is expected as they would be very common throughout mammals as a part of the regulatory process in the body.

Another set of similar proteins found abundantly throughout serum appeared as the 6 spots identified as Pregnancy Zone Protein and one spot identified as α-2-macroglobulin. These two proteins are glycoproteins which are strongly homologous to one another in structure which inhibit proteases in blood plasma. These inhibitors work by first binding to proteases through a ―bait" region which causes a conformational change in the inhibitors. This change initiates the second thiol-ester region to emerge and covalently bond with a nucleophile on the protease. This activity sterically hinders the protease from binding with other proteins thus decreasing the overall enzymatic activity (Petersen, 1993).

After eliminating the multiple spots of one protein, the single spots were reviewed. The first spot identified was angiotensinogen. Angiotensinogen is a precursor

protein that is part of the Renin-Angiotensin System (RAS), a multi-functional circulating system that helps regulate several organs. The scope and complexity of the RAS continues to expand as research delves further into the many layers of the body affected by RAS. The simplified view of RAS starts at renin secretion by the liver, which circulates throughout the body and reacts with angiotensinogen to form Ang I. Ang I is then converted to the active form (Ang II) by ACE. The RAS has now been expanded to include Ang III, Ang IV, Ang 1- 7, multiple ACEs, and even intracellular RAS covering both endocrine, paracrine, and intracrine functions. Because of the ubiquitous nature of the RAS and its multiple physiological regulatory functions, it was eliminated as a potential biomarker of kidney degradation. (Fyhrquist and Saijonmaa, 2008).

The next spot identified was inter-α-inhibitor H4 heavy chain which is a part of the generic inter- α -inhibitor family of plasma proteins. This family contains multiple polypeptide chains and a single, heavy chain designated I α IH4P with the rat PRR containing 6 repeats of a Gly-X-Pro motif in a collagen-like pattern (Soury et al., 1998). IAIH4P is produced in the liver where its production is triggered by acute, systemic inflammation. Little more is known about the exact physiological role this protein plays. A published 2008 study of human interstitial cystitis patients suggested I α IH4P as a biomarker of kidney damage (Canter et al., 2008). The data from this research provided only a tentative identification due to an overall ion score of 97 with only two peptide ion scores of 33 and 64. The low score for such an abundant protein lowers the validity of the identification and lowers the potential of I α IH4P as a potential biomarker without further validation data.

Another serum protein identified in this study as a potential renal injury marker was glutathione peroxidase (GP). Glutathione peroxidase is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. Extracellular glutathione peroxidase has been shown to be derived from the proximal tubules of the kidney, and can be secreted into the renal extracellular fluid and on into the blood (Whitin, et al. 2002). Therefore, at least from functional and structural data, GS is a good candidate for further biomarker pre-

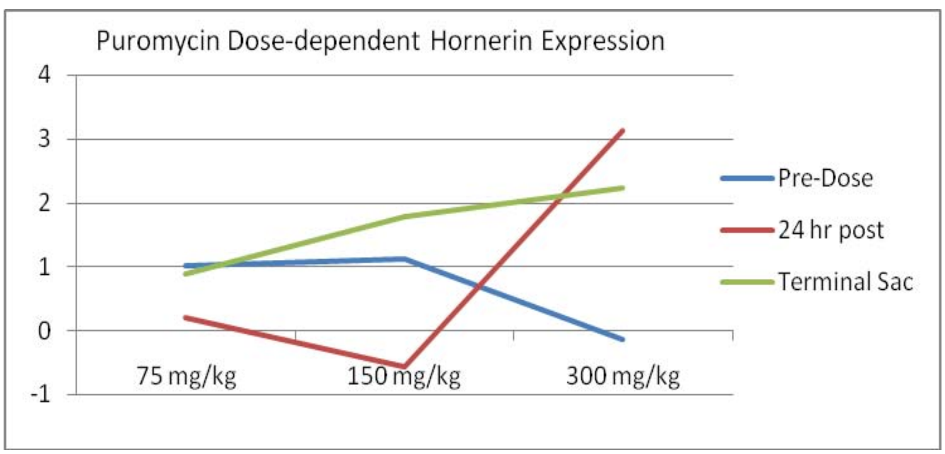
validation and validation. Glutathione peroxidase is a selenium-containing tetrameric glycoprotein molecule with four selenocysteine amino acid residues. The integrity of the cellular and subcellular membranes depend heavily on glutathione peroxidase, and the antioxidative protective system of glutathione peroxidase itself depends heavily on the presence of selenium. (Epp et al., 1983; Muller et al., 2007).

The final protein identified, hornerin, showed promise as this protein was also identified in ancillary work done in-house examining the urine proteome of nephrotoxin dosed animals (Shiyanov et al. 2009) with LC-MS/MS and ELISA work on rat urine samples from D-serine studies. In the serum proteome, the identification of this protein was judged ‗tentative' because of the low MALDI ion scores. Hornerin is a protein normally associated with epithelial cells so it is often used by other researchers as a sign of keratin contamination. Hornerin is a protein found routinely in epithelial cells and is typically listed as approximately 280 to 300 kDa in size. RHPB data using Western analysis has noticed a smaller protein band, approximately 55 kDa in size, consistently appearing in gel electrophoresis experiments but not been referenced in reviews of hornerin. This 55 kDa band has been reported to increase upon D-serine toxin exposures as early as 12 hours post dose (Mauzy et al. 2009). There is a lack of rat protein characterization on hornerin in DNA and protein databases. As the kDa size of the protein has been shown significantly lower than that of mouse or human and since the peptides clearly indicate homology to hornerin, this protein has been termed *"hornerin-like protein*" or HLP.

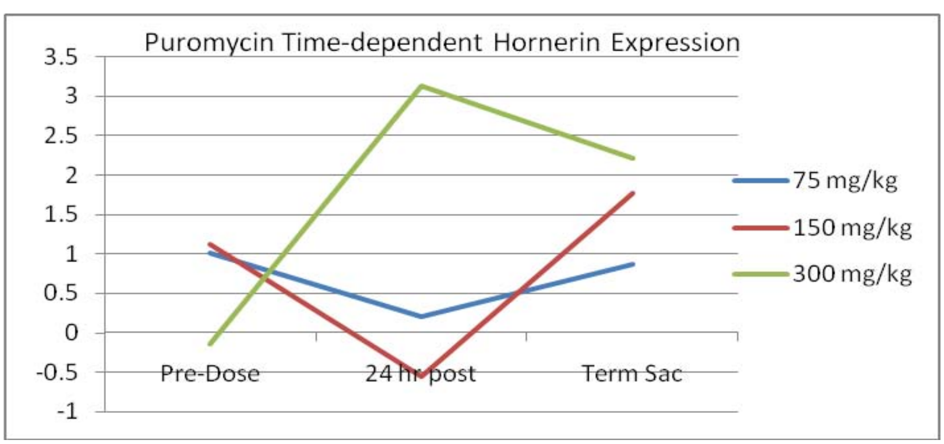
In general the X-fold increase data was not useful for determining dose or time dependent toxicity as the necessity of the use of pooled samples negated the ability to do appropriate statistics. If one of the animals had an abnormally high expression but four subjects were normal, the ‗averaging' of the differential protein expression could possibly indicate an increase that would be incorrectly attributed to all samples instead of the outlier. Despite the lack of significant statistics, the PG240 data revealed protein spot intensity trends that could indicate dose or time dependent toxicity. Immediately evident after analysis was the fact that the values for the differences in spot intensity were higher

for comparisons of ‗Control verses Control' gels than for later time points. That difference meant that the control samples were not consistent thus the normalized volumes could not be entirely trusted. While the dose dependent graphs for D-serine showed an increase in HLP as the dose increased and the time dependent graph showed a decrease in HLP from 12 hours to 24 hours (Figure 41F, 41G), HLP graphs in other nephrotoxin models showed useful trends for dose or time dependent analysis (Figure 41). Since the D-serine data from Study 2 was the only one to show expected trends, one must consider that the older serum samples from the puromycin and BEA animal studies were significantly degraded due to handling issues such as time and freeze/thaw cycles. Overall, the design of this research allowed for the narrowing of a broad field to a few viable biomarker candidates. Once the candidates have been identified, other studies could be constructed to identify dose and time dependent toxicity for the identified ‗potential' biomarker.

A



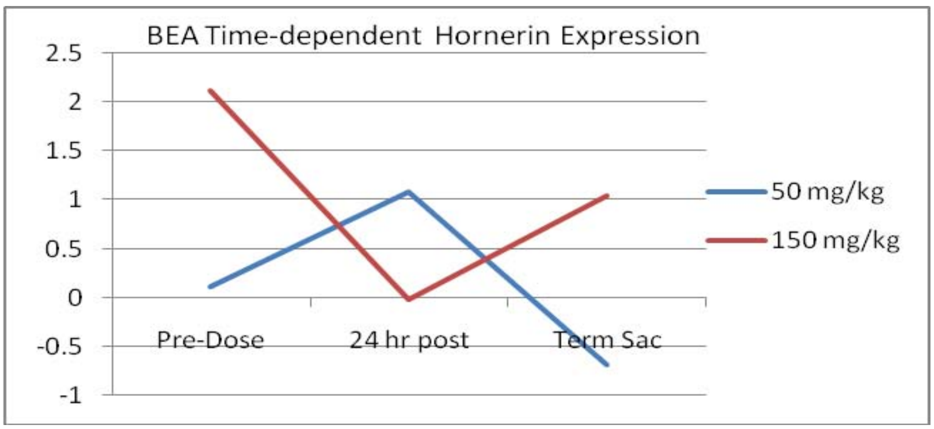
B



C

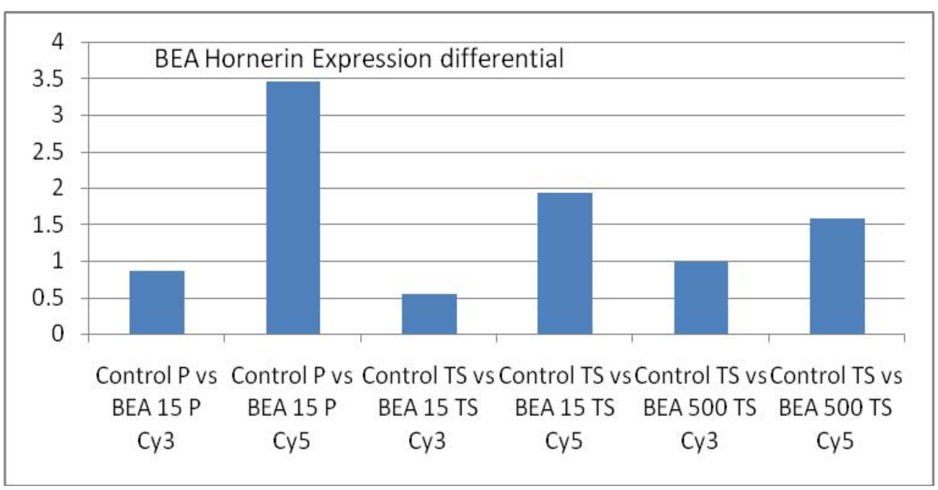
BEA Dose-dependent Hornerin Expression
3
2.5
2
1.5
1
0.5
0
-0.5
-1
15 mg/kg
50 mg/kg
150 mg/kg
500 mg/kg
Pre-Dose
24 hr post
Terminal Sac

D



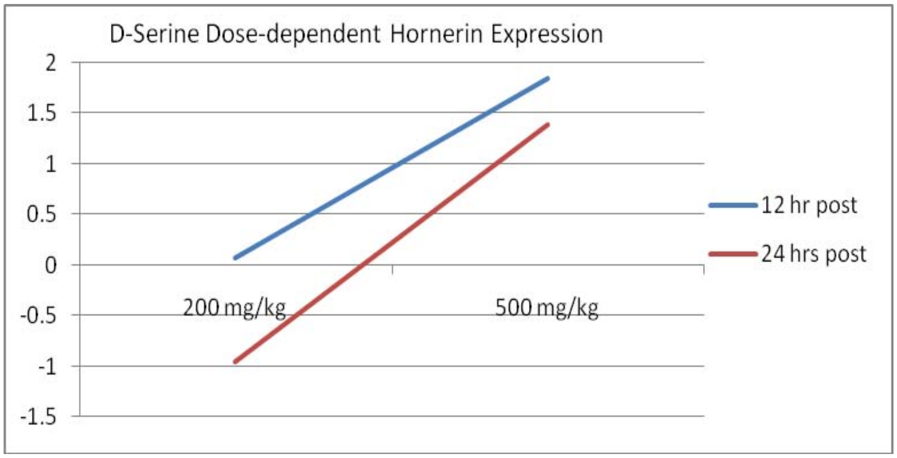
BEA Time-dependent Hornerin Expression
2.5
2
1.5
1
0.5
0
-0.5
-1
Pre-Dose
24 hr post
Term Sac
50 mg/kg
150 mg/kg

E



BEA Hornerin Expression differential
4
3.5
3
2.5
2
1.5
1
0.5
0
Control P vs BEA 15 P Cy3
Control P vs BEA 15 P Cy5
Control TS vs BEA 15 TS Cy3
Control TS vs BEA 15 TS Cy5
Control TS vs BEA 500 TS Cy3
Control TS vs BEA 500 TS Cy5

F



G

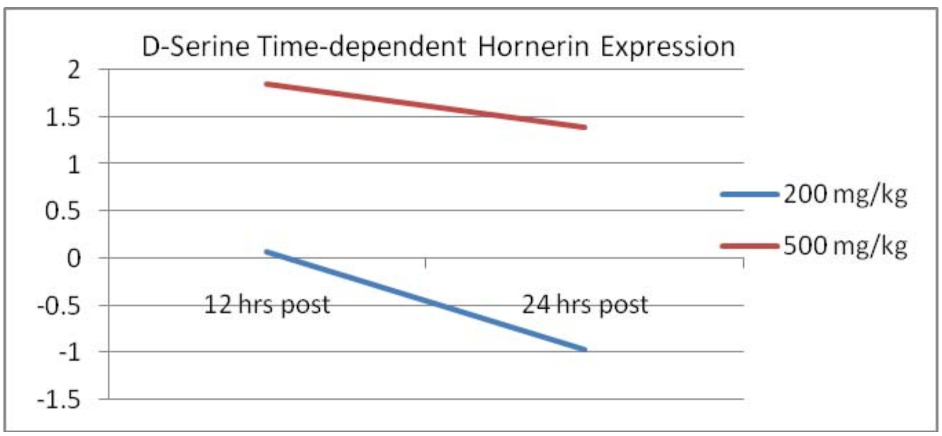


Figure 41: Graphs of the relative change in protein volume between the control sample versus the disease sample for each gel as time- and dose- increase.

The graphs show a general trend of increasing protein volume indicating greater amounts of the biomarker protein being produced as kidney functions decrease, either over time or from greater exposure to a nephrotoxin. Graphs: ***A)*** *Puromycin dose dependent hornerin expression* ***B)*** *Puromycin time-dependent hornerin expression* ***C)*** *BEA dose dependent hornerin expression* ***D)*** *BEA time dependent hornerin expression* ***E)*** *BEA hornerin expression differential P =predose, TS = terminal sacrifice,* ***F)*** *D-serine dose dependent hornerin expression* ***G)*** *D-Serine time dependent hornerin expression*

In conclusion, the proteins identified in this study were all well characterized, fairly abundant proteins known to exist throughout the body for multiple functions. The data identified two markers which may warrant further analysis as biomarkers of low level kidney degradation: HLP and glutathione peroxidase. Additional data generated

from other research in urine proteomics support the promising nature of HLP as a potential low level renal injury biomarker.

10. RECOMMENDATIONS

In order to determine dose or time dependent toxicity, further studies will be needed to be performed with sound statistical models. Further serum studies would require single animal subjects with control samples taken pre-dose followed by a single animal sacrifice at specific time points in order to collect enough serum for significant data points. This work indicated that 2D DIGE use is difficult in for research which produces small quantities of testable samples since too many variables are possible and the DIGE technique is not sensitive enough to quantitatively track such small concentrations. Therefore, it is not recommended for dose or time course studies with rats or smaller lab animals without the use of a terminal sacrifice at each time point. It may be valid for use in larger animals such as rabbits, guinea pigs, etc. where a larger blood sample volume may be drawn and stay within IACUC regulations. The methodology of this research demonstrated that, in the cases of serum proteomics utilizing low sample concentrations, a mass spectroscopy study such as an iTRAQ (isobaric tag for relative and absolute quantitation) or ICAT (isotope-coded affinity tags) may be more appropriate. Such new methodology is currently under development at RHPB.

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12. LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

ACN	acetonitrile
BUN	blood urea nitrogen
CAD	collisionally activated dissociation
CID	collisionally induced dissociation
CKD	chronic kidney disease
DIGE	2D Difference in Gel Electrophoresis
DTT	Dithiothreitol
E	Eluate (elution volume)
FAH	fumarylacetoacetate hydrolase
4-AP	4-aminopyridine
FT	flow through
GBM	glomerular basement membrane
GEC	glomerular epithelial cells
GFR	glomerular filtration rate
HIV	human immunodeficiency virus
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
IPG	isoelectric point gradient
kg	kilogram
kV	kilovolt
L	liter
LC-ESI MS/MS	Liquid Chromatography electrospray ionization tandem mass spectrometry
mA	milliamp
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-flight
MARS	Multiple Affinity Removal System for Mouse Serum
mL	milliliter
MS	mass spectrophotometry
mTorr	milliTorr
m/z	mass to charge ratio
NI	Non-interferring
PAN	Puromycin aminonucleoside
PCA	Principle Component Analysis
ppm	parts per million
PVDF	polyvinylidene fluoride
RBP	retinol binding protein
SARS	Severe acute respiratory syndrome
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered Saline
TBST	Buffer composed of Tris-Buffered Saline and Tween 20
uL	microliter
2D	Two dimensional